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SECTION MEETINGS

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Western Reserve University	February 19, 1959
WISCONSIN	
University of Wisconsin	December 18, 1958
MICHIGAN	
Wayne State University	January 15, 1959

Growth Promoting Activity of Butterfat Acids for *Lactobacillus casei* 280-16A.* (24652)

Merrill N. Camien and Max S. Dunn
Chemical Laboratory, University of California, Los Angeles

Lactobacillus casei 280-16A is a D-α-hydroxy acid requiring mutant strain derived from *L. casei* 7469(1,2).† Hydrolysates of butterfat and wool wax as well as any one of a number of synthetic D- or DL-α-hydroxy fatty acids support growth of this organism (3). Saponified wool wax contains D-α-hydroxy fatty acids(4,5) which account for its growth-promoting activity. Butterfat hydrolysates, on the other hand, are not known to contain D-α-hydroxy fatty acids. Isolation and identification of the growth promoting butterfat acids were therefore attempted as described here.

Methods. Saponified butterfat was prepared and assayed essentially as described previously(3). Amphetamine salts of the butterfat acids‡ were fractionated by a series of three 100-transfer distributions between ethyl acetate and 70% ethylene glycol (by volume) in water. Growth promoting potencies and relative concentrations of solutions from the countercurrent apparatus were determined by microbiological assays and optical density (at 260 mμ) readings, respectively. Amphetamine was removed from the final microbiologically active fraction by making the aqueous phase alkaline with sodium hydroxide and extracting the mixture with several portions of ether. The residual solution was acidified with hydrochloric acid, and its content of fatty acid was taken into ether. The ether solution was evaporated, and the fatty acid was crystallized from ethanol-water.

Results. The growth promoting fraction appeared to be nearly homogeneous after the

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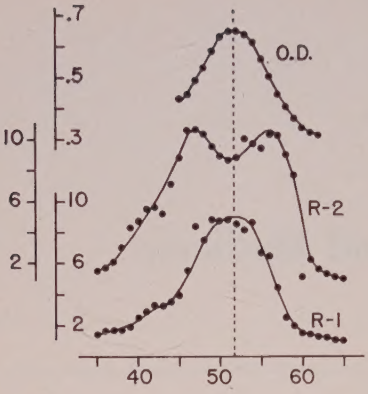
† Essential characteristics of strain 280-16A are the same as those originally described for strain 280-16(2,3).

‡ Prepared by mixing equivalent quantities of amphetamine (generously donated by Smith, Kline and French Labs) and butterfat acids. Amphetamine salts rather than free amino acids were employed because of convenience with which their relative concentrations may be determined photometrically.



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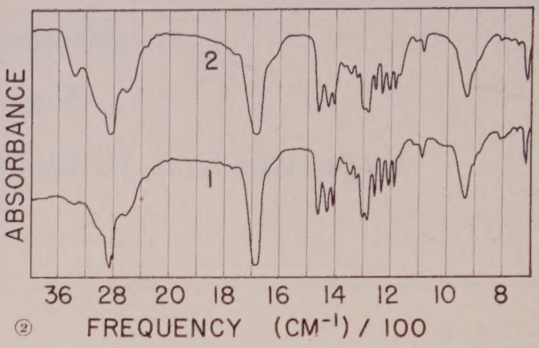

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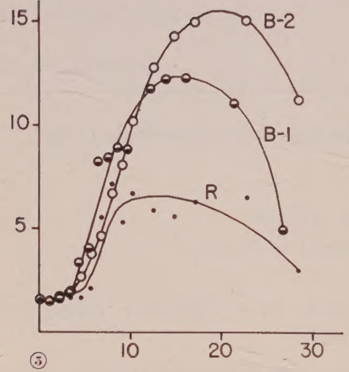

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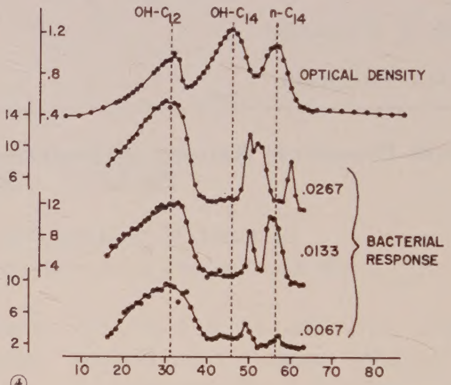

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FIG. 1. Distribution of microbiologically active butterfat acid (amphetamine salt) after 100 transfers in ethyl acetate/70% ethylene glycol (by volume in water). Values on the horizontal represent the countercurrent cell numbers. Values on the upper vertical scale (curve O.D.) represent optical density (260 mμ) of upper phase solution. Values on lower 2 vertical scales (curves R-1 and R-2) represent microbiological response (ml of 0.01 N NaOH required to titrate each ml of assay culture) to 13.3 μl and 26.6 μl, respectively, of upper phase solution/ml of assay medium.

FIG. 2. Infrared spectra of the microbiologically active butterfat acid (curve 1) and purified myristic acid (curve 2). Potassium bromide pellet preparations were employed.

FIG. 3. Response of *L. casei* 280-16A to the butterfat acid (curve B-1), the residue (m. 48-51°) from crystallization of butterfat acid (curve B-2), and a reference sample of purified myristic acid (curve R). Values on horizontal scale represent the test concentrations in μg/ml. Values on vertical scale represent response in terms of ml of 0.01 N sodium hydroxide required to titrate each ml of assay culture.

FIG. 4. Distribution of an artificial mixture of DL-α-hydroxylauric acid (OH-C_{12}), DL-α-hydroxymyristic acid (OH-C_{14}), and myristic acid (n-C_{14}) (amphetamine salts) after 100 transfers in ethyl acetate/70% ethylene glycol (by volume in water). Coordinates are same as in Fig. 1. Numbers shown on bacterial response curves indicate that .0067, .0133, and .0267 ml of upper phase solution were tested/ml of assay medium.

third countercurrent distribution, and the combined contents of cells 46-56 (Fig. 1) yielded what appeared to be essentially a single fatty acid. Approximately 86% of the acid was recovered as crystalline material melting at 52.6-53.2°, and the remainder as a semi-crystalline residue melting at 48-51°. Purified myristic acid (Eastman Kodak Co. white label product), when recrystallized from ethanol-water, melted in the same range (52.6-53.2°)§ as the isolated material, and its melting point was not depressed by mixing it with the latter substance. The infrared spectrum of the recrystallized myristic acid was

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§ Highly purified myristic acid melts at 54.4° according to Privett, *et al.*(6).

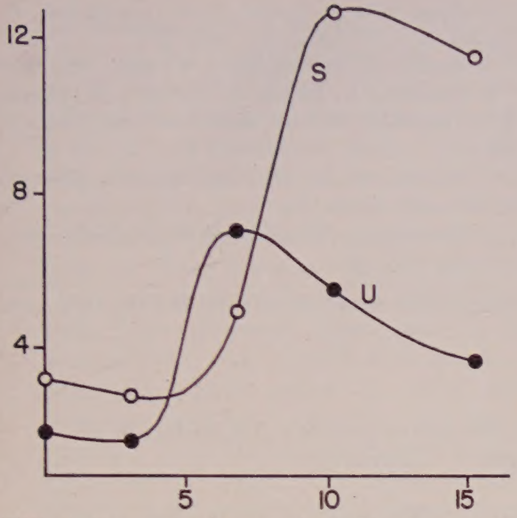

FIG. 5. Response of *L. casei* 280-16A to myristic acid in the unsupplemented assay medium (curve U) and in the same medium supplemented with 1.6 μg of DL-α-hydroxymyristic acid/ml. Coordinates are same as in Fig. 3.

essentially identical with that of the isolated butterfat acid (Fig. 2), however the latter product differed from the reference material by exhibiting pronounced growth promoting activity (Fig. 3).

These results suggested that the isolated material was essentially myristic acid but that traces of a contaminating acid accounted for its growth promoting activity. Such a contaminant would need either to possess an extremely high intrinsic growth promoting activity or to be capable of potentiating a latent growth promoting effect of myristic acid. The latter view was apparently substantiated by discovery of an analogous potentiating effect of α-hydroxymyristic acid when a artificial mixture of this substance with myristic acid and α-hydroxylauric acid was partially separated in a test of the resolving power of the countercurrent distribution system (Fig. 4). It may be seen (Fig. 4) that whereas α-hydroxymyristic acid was in itself without growth promoting activity,|| mixtures of it with myristic acid (overlapping distributions, Fig. 4) definitely promoted growth. The potentiating effect of α-hydroxymyristic acid was also apparent when tests of myristic acid activity were made in the test medium with and without a supplement of α-hydroxymyristic acid (Fig. 5). Inhibitory effects of myristic acid at elevated concentrations have been reported(7) and are evident from the data shown in Fig. 3 and 5. These inhibitory effects account for the dip in bacterial response corresponding to peak acid concentrations in Fig. 1.

Discussion. It may be inferred from these results that the growth promoting activity of saponified butterfat for *L. casei* 280-16A is due principally to myristic acid but depends also on potentiating effects of some other acid (or acids). α-Hydroxymyristic acid is an effective potentiating substance but has a considerably lower partition ratio than myristic acid (Fig. 4) and hence could not account for the symmetrical correlation between growth response and relative concentrations shown in Fig. 1. A monohydroxypalmitic acid, on the other hand, might be expected to have nearly the same partition ratio as myristic acid,¶ and it is of interest, therefore, that such an acid has been isolated from butterfat by Bosworth and Helz(8). Whether or not the monohydroxypalmitic acid of Bosworth and Helz has a potentiating effect similar to that of α-hydroxymyristic acid has not been determined.

Summary. A fatty acid which promoted growth of *L. casei* 280-16A was isolated from saponified butterfat and identified as myristic acid together with traces of an unidentified contaminant. The contaminant was apparently necessary to potentiate the growth pro-

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|| An irregular growth promoting activity has been reported for DL-α-hydroxymyristic acid(3), however analysis of the product (eq. wt. found 242.5 calculated 244.4) suggested contamination with myristic acid, which (it is now apparent) would result in a growth promoting mixture.

¶ Increase in partition ratio of a 14-carbon fatty acid effected by removing a hydroxy group (compare α-hydroxymyristic acid and myristic acid, Fig. 4) is apparently approximately equal to the decrease in partition ratio effected by removing 2 methylene groups (compare α-hydroxymyristic acid and α-hydroxylauric acid, Fig. 4). Little or no net change in partition ratio would be expected, therefore, upon removal of both a hydroxyl group and 2 methylene groups, as in going from a monohydroxypalmitic acid to myristic acid.

moting activity of myristic acid since purified commercial myristic acid (recrystallized) was essentially inactive. Activity of the latter could be potentiated by traces of α-hydroxymyristic acid, but this substance did not appear to be present in the isolated material.

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Trace Metal Requirements of *Aerobacter aerogenes* for Assimilation of Molecular Nitrogen.* (24653)

R. M. PENGRA AND P. W. WILSON

Bacteriology Dept., South Dakota State College, Brookings, South Dakota, and University of Wisconsin, Madison, Wis.

One of the surprising developments in biological nitrogen fixation during the past few years has been the discovery that many well-known bacteria previously unsuspected to possess the ability to use molecular nitrogen may do so when cultivated under appropriate conditions(1). These discoveries provide new tools for investigating the biochemistry of nitrogen fixation—studies that previously have been largely confined to the traditional agents of fixation: the azotobacter, the clostridia and the symbiotic system. For example, in spite of research extending over a quarter century, doubt still exists regarding the role of minerals (specifically, calcium, molybdenum and iron) in biological nitrogen fixation(2,3). Perhaps, some of the current disagreements in this respect can be resolved if attention were directed to the requirements of the new agents rather than concentration on the old. With this in mind, we have investigated the response of *Aerobacter aerogenes* strain 5al to these 3 minerals in the fixation of N_2.

Materials and methods. The growth medium and analytical procedures were essentially those previously described(2,4) altered as indicated for specific studies. The test medium for studies on the iron requirement was the same except that demineralized water prepared by passing distilled water through a Barstead "Bantamatic" demineralizer was used in all media, and iron was added separately rather than with the molybdenum solution. Solutions of the phosphate salts were run through 1 x 10 cm columns of Dowex 50 resin charged with sodium or potassium ions; a 5% solution of the corresponding chloride salt was used to charge the columns. Before passing the phosphate solutions through, the columns were washed with demineralized water until the effluent pH was near 7. A 10% solution of sucrose was purified by passing it through a similar column charged with hydrogen ions. The following solutions were prepared: Solution A, 12.5 g Na_2HPO_4 (Mallinckrodt, A. R. grade or comparable purity for all salts) in 250 ml; B, 1.5 g KH_2PO_4 in 250 ml water; C, 10 g Bacto-sucrose in 100 ml water; D, $MgSO_4 \cdot 7H_2O$ in 250 ml slurried with several grams of Dowex 50 charged with Mg ions; E. 0.02 g, $CaCl_2$ in 10 ml water; F. 2.5 μg molybdenum/ml as Na_2MoO_4; G. 2.5 μg iron/ml as $FeCl_3$. Combination of the solutions in the following quantities made up the media in the test flasks: 6.25 ml A, 6.25 ml B, 5.0 ml C, 2.5 ml D, 1 drop E, 1.0 ml F,

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and amounts of G to give 0.5, 0.1, 0.08, 0.06, 0.04, 0.02, 0.01, and 0.00 parts per million of iron in the series of flasks. Water was added to bring the volume of the medium to 25 ml in the 125 ml Erlenmeyer flasks. The flasks were covered with 50 ml beakers and autoclaved at 15 lb. pressure for 20 min. A 0.5% inoculum of *Aerobacter aerogenes* strain M5al from an iron deficient culture was used. When growth was followed turbidimetrically, flasks with colorimeter tubes fused to them were used(4). All cultures were grown on a Brunswick rotary shaker at 30°C, unless otherwise indicated. Nitrogen fixed was determined by a semi-micro Kjeldahl method (5). For the studies with carbon monoxide, growth of the cultures was followed by placing 50 ml of a one liter culture of the organism just entering the exponential phase of growth into each of several 500 ml Erlenmeyer flasks with pyrex glass tubes of a size to fit the Klett-Summerson colorimeter fused into the side. The desired atmospheres were placed in the flasks by evacuation with a water pump and refilling with the required gas mixture. Growth was followed turbidimetrically for 3 to 4 hours. For determination of molybdenum requirement, the medium was freed of this element by the method employed by Nicholas and Fielding(6) based on coprecipitation of the molybdate ion with a ferric quinolate complex from the macro-nutrients after which the excess quinolate is extracted from the solution with chloroform and ether. Adequate iron is then added to the medium together with the desired quantities of sodium molybdate solution. Inoculation was made from a molybdenum deficient culture, and after 3 to 4 days of incubation, the cultures were harvested and analyzed as has been described for the experiments on iron. Control flasks containing 150 or 200 μg of ammonium acetate-nitrogen were included in each experiment. The supply of ammonium acetate was checked for molybdenum by ashing a sample of the salt and adding the "residue" to control flasks. No fixation occurred in these flasks. For determination of the calcium requirement the 2 phosphate salts and the $MgSO_4$ were recrystallized from ion free water and the balance of the chemicals used were Analytical Reagent grade (Mallinckrodt). Inoculation, incubation and harvesting of the cultures were employed as has been described. Helium served as the atmosphere in the fixed nitrogen controls.

TABLE I. Inhibition of *Aerobacter aerogenes* by Carbon Monoxide.

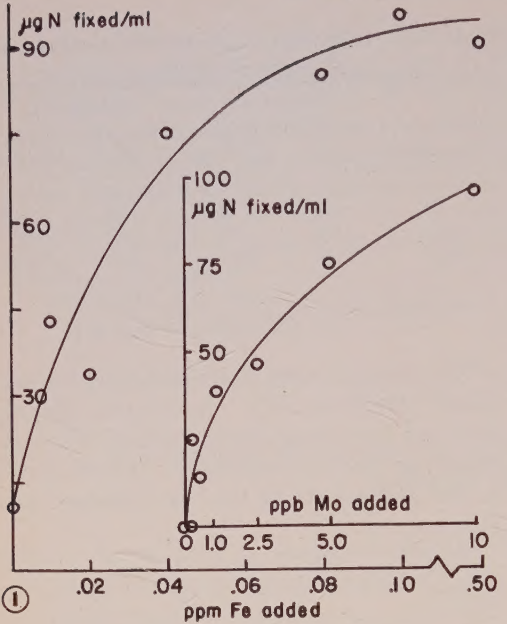
	Ammonia		Nitrogen gas	
% CO in atmosphere	Growth rate*	% inhibition	Growth rate*	% inhibition
.0	.200		.149	
.1	.202	.0	.098	34.2
1.0	.196	2.0	.057	68.5
10.0	.107	49.6	.000	100.0

* Growth rate $= \frac{2.3}{\text{Time in hr}} \log \frac{\text{Final N}}{\text{Initial N}}$.

Results. Cultures of *A. aerogenes* supplied with ammonium acetate as the nitrogen source and with an atmosphere of helium grew at the same rate and to the same extent when supplemented with 1.0, 0.5, 0.1, or 0.0 parts per million of iron, but as is shown in Fig. 1, nitrogen fixation is definitely stimulated by iron. This agrees with the finding of Esposito and Wilson (2) for *Azotobacter vinelandii* and with Carnahan and Castle(7), working with the anaerobic nitrogen fixer *Clostridium pasteurianum*.

Although growth on both free and fixed nitrogen was inhibited by carbon monoxide, assimilation of molecular nitrogen was about one hundred times as sensitive to carbon monoxide as was assimilation of ammonia nitrogen (Table I). Although carbon monoxide inhibition of a biological reaction is not *per se* proof that iron is involved in the reaction, demonstration of such an inhibiton provides support for the view that iron is involved. In comparison, Wilson and Lind(8) observed CO inhibition of nitrogen fixation by *Azotobacter vinelandii*, and Hino(9) observed CO inhibition of fixation by an organism he believed at the time to be a member of the genus *Clostridium*, but now shown to be a species of *Bacillus*(10).

The quantity of molybdenum required for maximum growth on N_2 was so small that some difficulty was encountered in obtaining a response curve but finally the curve insert in Fig. 1 was obtained. No requirement for



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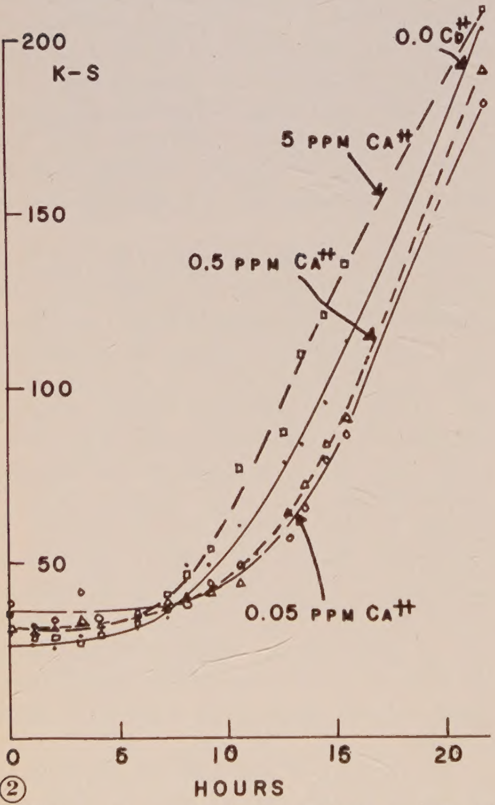

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FIG. 1. Response of *A. aerogenes* to iron and molybdenum when assimilating free nitrogen.

FIG. 2. Effect of calcium concentration of nitrogen fixation by *A. aerogenes*. K-S is Klett-Summerson colorimeter readings.

molybdenum could ever be demonstrated when the organism was growing on ammonium nitrogen. Esposito and Wilson(2) demonstrated a definite requirement for molybdenum by nitrogen fixing cultures of azotobacter. Their data does not allow one to decide the minimum amount required for fixation, but it appears to be somewhat lower than the iron requirement.

Although Esposito and Wilson(2) observed a definite stimulation in nitrogen fixation by *Azotobacter vinelandii* on addition of calcium, Norris and Jensen(3) claim that the requirement is not specific for fixation. The results with the strain of *Aerobacter aerogenes* used in this work likewise did not reveal a requirement for calcium (Fig. 2). Perhaps the discrepancies reported by various investigators can be explained in part by the more recent work of Esposito and Wilson(11) that revealed that the calcium requirement specific for fixation in *A. vinelandii* O can be replaced by acetate. The implication is that calcium is not involved directly in nitrogen fixation but in the production of an essential carbon compound(s). If this is the true explanation of its action, variation would be expected among different agents, even extending to strains, such as has been observed by the various investigators.

Summary. *Aerobacter aerogenes* strain M5al fixing molecular nitrogen require at least 10 times more iron than do cultures using ammonium-N. Also, the organism is about 100 times more sensitive to carbon monoxide when using free than when assimilating combined nitrogen. Although there is an absolute requirement for molybdenum for nitrogen fixation by this organism, it is considerably less than that noted for many strains of the azotobacter. No specific requirement for calcium could be demonstrated.

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Effect of Estrogen on Liver Glycogen in Adrenalectomized Rats.* (25654)

DWIGHT J. INGLE
Ben May Laboratory for Cancer Research, University of Chicago, Ill.

Large doses of injected estrogen cause increased glycogen in livers of intact fasting rats but not in livers of untreated adrenalectomized rats(1,2). It was concluded that the glycogenic activity of estrogen is mediated by increased secretory activity of the adrenal cortex. The present study demonstrated an extra-adrenal effect of estrogen upon level of glycogen in livers of adrenalectomized rats treated with adrenal cortical extract (ACE).

Methods. Male rats of the Sprague-Dawley strain weighing approximately 300 g were adapted to a medium carbohydrate diet(3), tube-fed each morning and late afternoon. The temperature was 74° to 78°F. Adrenalectomies and sham adrenalectomies were done by sterile technic under ether anesthesia. The adrenalectomized rats were treated with ACE (Upjohn) having glycogenic potency of 0.1 mg of hydrocortisone/cc. Whole adrenal cortical extract was used because it represents more complete replacement therapy than any single steroid. From fifth postoperative day, estradiol cyclophentyl-propionate (Upjohn) in oil solution (1 mg/cc) was injected subcutaneously in 0.1 mg daily for 4 days. Each rat was then fasted 24 hours and, under sodium cyclopal anesthesia, the liver was rapildly excised, weighed, and dropped into hot KOH. Glycogen was determined by the anthrone method(4). Since each rat had the same initial weight and was given the same amount of food, the results are expressed as total amount of liver glycogen/rat.

Results. Five experimental groups of 60 rats each were studied in relationship to dose of ACE given the adrenalectomized rats. Each group was divided into 4 equal subgroups: adrenalectomized, no estrogen; adrenalectomized, estrogen; adrenals intact, no estrogen; and, adrenals intact, estrogen. Each subgroup was equally represented in time. The values on liver glycogen are in Table I. Livers of rats treated with estrogen averaged 21% heavier than livers of rats not given estrogen. The average amount of glycogen recovered from liver varied somewhat, but in the non-adrenalectomized rat treated with estrogen a significantly greater amount was always found. Among rats not given estrogen, the average amount of liver glycogen in adrenalectomized rats was less than in intact rats until 6 cc of ACE/rat/day were given. The injection of estrogen was associated with increased liver glycogen in each subgroup of adrenalectomized rats, the amount increasing as dose of ACE was increased. Even with a dose of 6 cc of ACE daily, the adrenalectomized rats given estrogen showed significantly smaller amounts of liver glycogen than did the non-adrenalectomized rats given estrogen.

Discussion. Estrogen has an extra-adrenal effect on amount of liver glycogen in the rat. The mechanism of this effect is unknown. It is possible than increased secretion of cortical hormones by the estrogen-treated intact rat

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* This research was supported by grant from Eli Lilly Co., Indianapolis, Ind.

TABLE I. Effect of Estradiol on Liver Glycogen in Intact Rats and in Adrenalectomized Rats Treated with Adrenal Cortex Extract. Fifteen rats/group.

ACE, cc/day	Adrenalectomy Liver glycogen, mg/rat No estrogen	 Estrogen	Adrenals intact, no ACE Liver glycogen, mg/rat No estrogen	 Estrogen
1	9.5 ± .52	12.3 ± .21	16.6 ± 2.11	105.3 ± 8.30
2	8.7 ± .71	35.0 ± 3.27	20.5 ± 2.97	76.6 ± 6.23
4	9.5 ± .95	39.7 ± 3.07	16.6 ± 1.88	89.0 ± 6.41
5	13.4 ± .76	51.1 ± 4.44	18.2 ± 1.76	72.3 ± 5.08
6	28.4 ± 4.37	62.4 ± 5.11	19.7 ± 2.31	93.8 ± 7.20

enhances deposition of liver glycogen; response to estrogen was greater in the presence of adrenals. It is also possible that estrogen has no glycogenic effect of its own but causes a rise in titer of cortical hormones by suppressing rate of destruction of these steroids. Mills(5) has published evidence that such can occur.

The principal effect of the cortical hormones in supporting the glycogenic effect of estrogen may be a permissive one. The term "permissive" was first used to call attention to biological responses which fail to become overt in the adrenally insufficient animal but do occur in adrenalectomized animals maintained in a state of eucorticalism by a steady intake of cortical hormones. It is assumed that the permissive role of a hormone represents normalizing the functions of organs—sustaining the integrity of metabolic processes and thereby the capacity to adapt and respond. A large number of biological responses are now known to require the presence of cortical hormones but not the adrenal glands. The term "permissive," although still useful, covers our ignorance of several mechanisms by which hormones and other biological principles support homeostasis and adaptability.

Summary. Among 300 force-fed male rats of the Sprague-Dawley strain (300 g initial wt.), estrogen increased liver glycogen in the fasting adrenalectomized rat treated with ACE whereas this effect was not observed in the adrenally insufficient animal. The glycogenic effect of estrogen was greater in intact rats than in adrenalectomized rats treated with ACE.

Acknowledgement is made of the technical assistance of Mr. Mitchell Heller and Mr. Melvin Vinik on some experiments.

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Effect of Dietary Iron on Phosphate Metabolism.* (24655)

WOLFGANG BUTTNER† AND JOSEPH C. MUHLER
Dept. of Biochemistry, Indiana University Medical Center, Indianapolis

In experiments involving the effect of different carbohydrates on the calcification process, differences were observed that could not be accounted for by any readily known mechanism. Upon further scrutiny of the dietary components as well as the experimental procedures, it was found that the mineral supplement used in these studies had an unusually large iron content. This observation prompted us to design a study in which the amount of

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* This study was supported in part by grant from Procter and Gamble Co., Cincinnati. O.

† Post-doctorate fellow. Present address: Univ. of Mainz, Germany.

TABLE I. Effect of Extra Dietary Iron and Phosphate on Calcium and Phosphorus Content of Newly Formed Dentin of Rat Incisors and of Iron Content of Liver.

	Ca and P content of apical dentin in incisor teeth			Fe in livers	
Group	Ca, mg %	P, mg %	Ca:P	Total, mg	Conc., mg %
I	26.9	13.5	1.99	.9 ± .1*	38 ± 7
II	27.6	13.7	2.01	5.9 ± .5	210 ± 40
III	28.0	14.4	1.94	2.9 ± .3	120 ± 20
IV	23.8	12.5	1.90	2.8 ± .3	100 ± 10
V	26.7	13.8	1.93	.6 ± .1	25 ± 3
VI	25.6	13.8	1.86	.9 ± .2	50 ± 8

I = control; II = 50 mg Fe; III = 50 mg Fe + 50 mg P; IV = 50 mg Fe + 100 mg P; V = 50 mg P; VI = 100 mg P.

* Stand. dev.

phosphate in calcified tissues was evaluated when extra dietary levels of iron were administered to rats.

Experimental. A total of 48 male Sprague-Dawley strain rats, weighing between 92 and 108 g, were divided equally into 6 experimental groups according to their body weight. All animals received a stock corn diet and redistilled water *ad lib.*, the calcium and phosphorus content of which was 0.32 and 0.31 mg % respectively. The animals in Group I received only this diet. Group II animals received 50 mg Fe, those in Group III 50 mg Fe plus 50 mg P, and those in Group IV 50 mg Fe plus 100 mg P. Animals composing Groups V and VI received 50 and 100 mg P, respectively. Fe was administered as $FeSo_4$ and P as NaH_2Po_4, and both were given simultaneously by stomach tube daily throughout a 3 week period. After termination of the study period, the animals were sacrificed and the livers were removed and analyzed for iron content. The maxillary central incisors were also removed and the newly formed dentin removed and analyzed for calcium and phosphorus. All animals were housed separately in raised screen cages in an air-conditioned room.

Results. The data obtained in this study are found in Table I. Calcium content of the incisor dentin is higher in the group receiving Fe or Fe plus 50 mg P, but significantly lower ($p = .02$) when Fe plus 100 mg P is administered. When 50 mg P and no Fe is given, calcium content of incisor dentin is not affected, but when 100 mg P is administered there is less calcium in the dentin ($p = .06$). Phosphorus content of incisor dentin does not seem to be nearly as variable as calcium content and is higher when Fe plus 50 mg P is administered ($p = 0.09$) and significantly less when Fe plus 100 mg P is given ($p = 0.05$).

Iron content of liver shows the effect of extra dietary iron therapy. There is a 6 fold increase in iron content of livers of the group receiving 50 mg Fe when compared to controls. However, when 50 mg Fe plus 50 mg P is administered there is a highy significant ($p = 0.0001$) reduction in total liver iron content when compared to the group receiving the same amount of Fe but no added phosphate. Similar findings result when 50 mg Fe plus 100 mg P is given. No significant differences in liver iron content result when P is administered in absence of extra dietary iron.

Discussion. Increasing the dietary iron content significantly increases iron content of the liver. However, as the phosphate content of the diet is similarly increased, liver iron content is reduced, apparently by forming an insoluble iron-phosphate complex which decreases the mutual metabolic availability of both iron and phosphorus. These results are similar to those of Hegsted, Finch and Kinney (1) who reported that the amount of iron deposited in the liver of rats is inversely related to phosphorus content of the diet. Of considerable interest, however, are the results obtained by the incisor dentin analysis in relation to extra dietary iron and phosphorus content. When 100 mg phosphate was administered in the presence of iron, a significant reduction in incisor dentin calcium and phosphorus content was found. Apparently, the concentration of iron and phosphate at this level is sufficient to modify significantly the blood phosphorus content to cause a reduction in concentration of both calcium and phosphorus in the growing dentin. An analysis of blood phosphorus content of these animals corroborates this finding in that a 20% reduction in blood phosphorus level was found when compared to control animals. These data would suggest that more study be given to those factors which complex both dietary iron and phosphate and which affect their absorption from the GI tract in relation to their effect on the calcification process in general.

Conclusions. Increasing the amount of iron by means of stomach tubing $FeSo_4$ to rats significantly increases liver iron content. When 50 mg phosphate is given simultaneously with iron, a significant reduction in liver iron content results, as well as a significant diminution in calcium and phosphorus content of newly forming incisor dentin. These results, compared with a significant reduction in blood phosphorus concentration, suggest that increasing the iron content of the diet for prolonged periods of time without significantly increasing the dietary phosphorus content may result in improperly calcified tissues.

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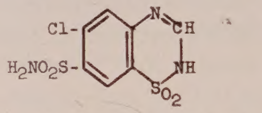
Saluretic Activity of Hydrochlorothiazide (6-Chloro-7-sulfamyl-3,4-dihydro-1,2,4-benzothiadiazine-1,1-dioxide) in the Dog. (24656)

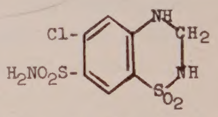
JOHN E. BAER, HORACE F. RUSSO AND KARL H. BEYER
Merck Inst. for Therapeutic Research, West Point, Pa.

Chlorothiazide,* first synthesized by Novello and Sprague(1), was shown to be a remarkably safe and potent agent for increasing excretion of sodium and chloride in animals (2,3) and in man(4). The agent has found widespread use in treatment of congestive failure, edema of pregnancy, premenstrual tension and other edematous states, and in management of hypertension. During research leading to discovery of the effectiveness of chlorothiazide as a saluretic-diuretic agent, many compounds more or less closely related to it in structure have been synthesized by Novello and Sprague and have been evaluated in these laboratories. We wish to report the preclinical evaluation of one such compound, hydrochlorothiazide,† that resembles chlorothiazide qualitatively but is considerably more potent. The structural formulae for the 2 compounds presented below make it evident that the new agent differs from chlorothiazide only in saturation of the heterocyclic portion of the molecule.



6-Chloro-7-sulfamyl-1,2,4-benzothiazine-1,1-dioxide

Chlorothiazide
Diuril*



6-Chloro-7-sulfamyl-3,4-dihydro-1,2,4-benzothiadiazine-1,1-dioxide

Hydrochlorothiazide
HydroDiuril†

Methods. Renal clearance studies were performed on trained unanesthetized female mongrel dogs in post-absorptive state. Water (500 ml by gavage) and isotonic mannitol-phosphate infusion at 3 ml/min. throughout experiment, assured adequate urine flow. Hydrochlorothiazide was introduced into the infusion during the "drug phase" of experiments at dosages indicated. Clearance of exogenous creatinine, administered subcutaneously, was used as a measure of glomerular filtration rate. Blood was collected at midpoint of each 10-minute clearance period. The powdered drug was given to dogs orally in gelatin capsules containing the solid compound. Fasting female mongrel dogs were intubated with 500 ml of water and urinary bladders were emptied by catheter. The animals were held in metabolism cages, and complete urine collections were made at regular intervals up to 6 hours. Hydrochlorothiazide was hydrolyzed in neutral solution (15 lb pressure for ½ hour) to a substance that can be measured by diazotization and coupling

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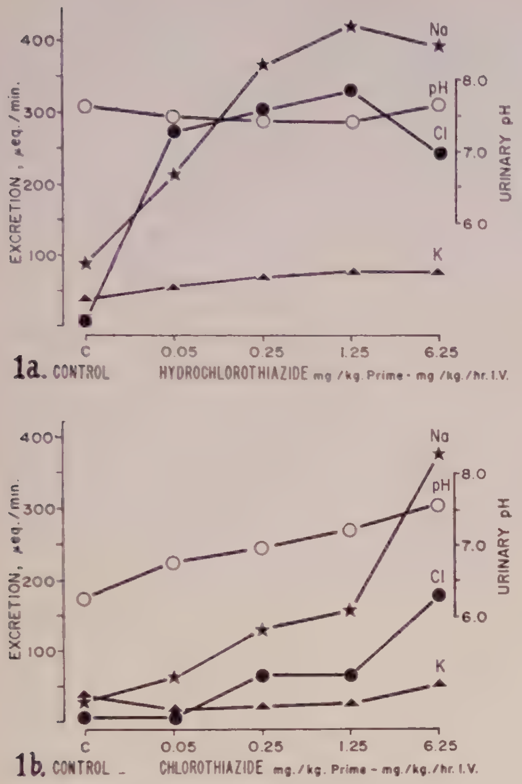
* DIURIL is trade-mark of Merck & Co. for its brand of 6-chloro-7-sulfamyl-1,2,4-benzothiadiazine-1,1-dioxide, the nonproprietary name of which is chlorothiazide.

† HYDRODIURIL is trade-mark of Merck & Co., for its brand of 6-chloro-7-sulfamyl-3,4-dihydro-1,2,4-benzothiadiazine-1,1-dioxide, the nonproprietary name of which is hydrochlorothiazide.



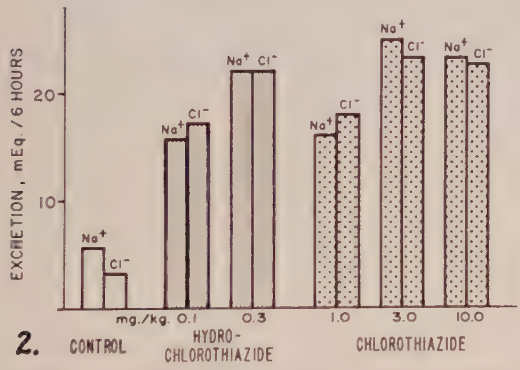


FIG. 1. Dosage response curves for (A) hydrochlorothiazide and (B) chlorothiazide on excretion of sodium, chloride, potassium and urinary pH in dogs. Duplicate successive 10-min. clearances were measured 20 min. after i.v. prime and onset of infusion at the indicated dosage level.

FIG. 2. Sodium and chloride excretion following single oral dose of hydrochlorothiazide or chlorothiazide in dogs. Avg 6-hr excretion for 6 dogs.

with N-1-naphthylethylenediamine; incorporation of this step in the procedure devised for chlorothiazide(5) permits estimation of higher concentrations of hydrochlorothiazide, but is not applicable at minimal plasma concentration required to produce saluresis.

Results. Hydrochlorothiazide caused a marked and equivalent increase in sodium and chloride excretion in dogs at lowest intravenous dosage shown in Fig. 1A. There was a lesser effect on potassium excretion even at highest dosage given, and urine pH did not reflect any alteration in bicarbonate excretion. Saluresis first increased, then reached a maximum and even decreased slightly as dosage of hydrochlorothiazide was increased to supramaximal level. In a similar experiment with chlorothiazide (Fig. 1B), little saluresis was observed at 0.05 mg/kg and maximal saluresis had not been attained at 6.25 mg/kg. Furthermore, sodium excretion exceeded that of chloride at higher doses of chlorothiazide, with resulting increase in bicarbonate excretion and in urine pH.

Hydrochlorothiazide and chlorothiazide, when given separately to a single dog at 0.05 and 0.25 mg/kg, intravenously, produced effects shown in Table I, from which it may be estimated that hydrochlorothiazide is 5 times as potent a natriuretic agent as chlorothiazide by this route in dogs. The lowest dose of hydrochlorothiazide caused an increase of 199 microequiv./min. of sodium and 163 microequiv./min. of chloride, whereas chlorothiazide caused corresponding increases of 68 and 10 microequiv./min. The increase in cation excretion that resulted from increasing the dose of hydrochlorothiazide was essentially covered by commensurate increase in chloride excretion. In the case of chlorothiazide, however, a portion of the cation was interpreted to have been covered by an increased bicarbonate excretion (increase in urine pH). The kaliuretic effect in these experiments was the same for both compounds.

The unusual effectiveness of chlorothiazide under experimental conditions of acidosis or alkalosis is duplicated by hydrochlorothiazide. The effect of small intravenous doses of hydrochlorothiazide on electrolyte excretion was determined in dogs that received 100 meq. of ammonium chloride or sodium bicarbonate daily for 5 days prior to clearance experiments shown in Table II. On sixth day, triplicate 10-minute clearances were obtained,

TABLE I. Comparative Effects of Increasing Intravenous Doses of Hydrochlorothiazide and Chlorothiazide in Dog 849.*

I.V. dose, mg/kg prime and mg/kg/hr infusion	Excretion, μequiv./min.			Na^+, % of filtered load reabsorbed†	Urine	
	Na^+	K^+	Cl^-		ml/min.	pH
Hydrochlorothiazide						
0	46	27	6	99.5	3.9	6.2
.05	245	47	169	97.4	2.9	7.4
.25	382	60	333	95.6	4.4	7.3
Chlorothiazide						
0	10	25	7	99.9	2.8	5.8
.05	78	49	17	99.1	2.7	7.4
.25	339	65	192	96.2	4.5	7.7

* Data are averages for duplicate successive 10-min. clearance periods.

† Calculated for each clearance period from measured plasma and urine concentration and glomerular filtration rate (creatinine clearance).

followed by 0.25 mg/kg priming dose and infusion of hydrochlorothiazide at 0.3 mg/kg/hr. for 20-minute equilibration period, after which 3 "drug phase" clearances were measured. In acidotic dogs (plasma HCO_3^- 10.7 and 11.5 meq./l), the increase of over 250 microequiv./min. in sodium excretion was more than covered by increase in chloride excretion. Urinary pH decreased and there was no appreciable excretion of bicarbonate. Duplicate experiments in the 2 animals were in good agreement and agreed well with similar experiments at 10 times the dose of chlorothiazide(6).

Control data for dogs that received sodium bicarbonate show that they were excreting large amounts of sodium and bicarbonate as a consequence of large dietary intake of these

TABLE II. Effect of Hydrochlorothiazide and Chlorothiazide on Electrolyte Excretion in 6 Dogs Made Acidotic with Ammonium Chloride or Alkalotic with Sodium Bicarbonate.*

	Dose		Sodium		Potassium	Chloride	Bicarbonate	Urine pH	Plasma pH	GFR,* ml/min.
	Prime, mg/kg	Infusion, mg/kg/hr	Excretion, μeq/min.	% of filtered load reabsorbed	Excretion, μeq/min.					
				Ammonium chloride						
Control			11	99.8	15	70	.3	5.2	7.26	45.4
Hydrochlorothiazide	.25	.3	305	95.2	62	425	6	4.9		45.1
Control			21	99.8	12	96	6	5.2	7.24	59.7
Hydrochlorothiazide	.25	.3	289	96.1	63	437	6	4.7		51.2
Control			18	99.8	34	80	2	5.3	7.23	57.5
Chlorothiazide	2.5	3.0	200	97.5	53	320	6	4.7		57.1
				Sodium bicarbonate						
Control			523	96.0	87	25	432	8.0	7.48	80.1
Hydrochlorothiazide	.25	.3	883	93.0	111	365	552	7.8		77.4
Control			220	97.3	78	7	209	7.9	7.49	55.1
Hydrochlorothiazide	.25	.3	727	92.8	142	316	389	7.8		68.1
Control			212	97.8	38	9	182	7.9	7.57	64.8
Chlorothiazide	2.5	3.0	598	92.8	78	194	415	8.0		55.5

* Ammonium chloride or sodium bicarbonate was administered at oral dose of 100 meq/day for 5 days; a renal clearance experiment was then performed; GFR = glomerular filtration rate.

Tabulated values are avg of triplicate successive 10-min. clearance periods.

TABLE III. Renal Clearance of Hydrochlorothiazide in the Dog.

	Urine		Hydrochlorothiazide			
Time, min.	ml/min.	pH	Plasma conc., mg/l	Clearance, ml/min.	GFR, ml/min.	Clearance ratio
0		3 g creatinine, subcut.				
10		Mannitol-phosphate infusion, 3 ml/min.				
30–40	.2	6.4			41.9	
40–50	.5	6.5			52.7	
	Hydrochlorothiazide: 2.5 mg/kg prime, i.v.; 3.0 mg/kg/hr					
80– 90	5.5	7.3	2.7	213	46.1	4.61
90–100	6.2	7.3	2.9	175	43.6	4.02
	Probenecid: 25 mg/kg prime, i.v.; 30 mg/kg/hr					
120–130	4.0	7.0	4.4	25	33.3	0.75
130–140	4.3	6.9	5.2	26	36.0	0.74

ions. Plasma bicarbonate was elevated (26.7 and 27.2 meq./l), urine was alkaline, and chloride excretion was minimal. Hydrochlorothiazide produced a marked increase in electrolyte excretion in alkalotic dogs. The greatest absolute increase was noted for sodium ion, although reduction in "percentage of filtered load reabsorbed" was similar to that noted in acidotic dogs. The already large bicarbonate excretion was increased somewhat, but chloride excretion rose from a negligible rate to cover nearly two-thirds of the increase in cation excretion. Duplicate experiments in 2 animals were in good agreement with each other and with similar experiments wherein a 10-fold greater dose of chlorothiazide was employed(6).

Hydrochlorothiazide caused an enhanced excretion of sodium and chloride during 6-hour period in dogs that received 0.1, 0.3, 1, or 3 mg/kg orally. A less than maximal saluretic response was obtained only at dosage less than 0.3 mg/kg. With chlorothiazide, a submaximal saluresis occurred at 1 mg/kg, and the response obtained at 3 mg/kg was maximal. Fig. 2 shows average sodium and chloride excretion for groups of 6 dogs at several significant dosages of the 2 compounds. The response to 0.1 mg/kg of hydrochlorothiazide resembled that observed at 1 mg/kg of chlorothiazide. Hydrochlorothiazide was uniformly well absorbed, as judged by the saluresis that invariably followed its oral administration to the animals.

Renal clearance of hydrochlorothiazide exceeds glomerular filtration rate (Table III). To obtain a plasma concentration of hydrochlorothiazide that could be determined analytically, a much larger dosage of hydrochlorothiazide was employed than is required to produce saluresis. This relatively large dose of the agent did not affect glomerular filtration rate, as measured by exogenous creatinine clearance. Hydrochlorothiazide itself was excreted at 4 times glomerular filtration rate, the large renal clearance indicating secretion of the drug by renal tubules. Administration of probenecid[‡] in the third phase of experiment, reduced clearance of hydrochlorothiazide (uncorrected for plasma binding) to slightly less than glomerular filtration rate.

Discussion. Enhanced saluretic activity that results from saturation of the heterocyclic ring of chlorothiazide appears to be consequent to an increase in potency, rather than to a qualitatively different type of activity. Thus, no really qualitative difference is seen in the spectrum of electrolyte excretion of chlorothiazide and hydrochlorothiazide over the more than 100-fold dosage range shown in Fig. 1. Likewise, in severe acidosis or alkalosis, as induced by ammonium chloride or sodium bicarbonate ingestion, the electrolyte excretion pattern, and in fact the actual data, for hydrochlorothiazide (Table II) resemble closely those of experiments at 10 times the dose of chlorothiazide(6). Similarly, in oral experiments, a half-maximal excretion of sodium is accompanied by equivalent chloruresis, but this response is obtained at a dosage of 0.1 mg/kg of hydrochlorothiazide and 1 mg/kg of chlorothiazide.

[‡] BENEMID is trade-mark of Merck & Co., for its brand of p-(di-n-propylsulfamyl)-benzoic acid, the nonproprietary name of which is probenecid.

Both chlorothiazide and hydrochlorothiazide are secreted by renal tubules, being cleared at rates in excess of glomerular filtration rate. Both compounds, thus, will be removed rapidly from blood of normal dogs following a single intravenous dose. The mechanism whereby both chlorothiazide and hydrochlorothiazide are excreted in excess of glomerular filtration presumably involves that utilized by penicillin, p-aminohippurate and certain other organic acids, inasmuch as it is suppressed by administration of probenecid, an agent known to depress selectively renal tubular secretion of certain organic acids without affecting electrolyte excretion and without interference in renal transport of numerous other substances(7).

Summary. Hydrochlorothiazide is a potent saluretic agent. It resembles chlorothiazide qualitatively, but is several times more active, intravenously and orally, in the dog.

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A Metabolite of 1-Hydrazinophthalazine (Hydralazine).* (24657)

Carl D. Douglass and Rose Hogan
Dept. of Biochemistry, School of Medicine, University of Arkansas, Little Rock

The antihypertensive drug 1-hydrazinophthalazine (hydralazine, apresoline) has been shown to interfere in the process of biological acetylation of sulfanilamide(1). The mechanism for this interference appeared to be competition between substrate and drug for the active acetyl group. It was further shown that free hydralazine disappeared from pigeon liver extracts when all components of the acetylation system were present. These facts suggested the possibility that 1-acetyl-2-phthalazyl hydrazine (acetyl hydralazine) should be formed as a conjugate of hydralazine. We here report the identification of this material as a product of hydralazine metabolism *in vitro* and *in vivo*.

Materials and methods. In vitro incubations and extractions. An adult pigeon was killed by decapitation and its liver rapidly dissected out and placed in cold Robinson's solution(2) containing 20 mg of glucose per 100 ml. Ten grams of liver slices were prepared with the Stadie-Riggs microtome. The slices were divided into 2 equal portions and each washed twice with Robinson's solution and finally suspended in 10 ml of the solution containing 200 μmoles of sodium acetate and in one case 100 μmoles of hydralazine. The latter materials had been previously made up in a small portion of Robinson's solution and neutralized before incroporation into the suspending medium. The mixtures were incubated with shaking at 37° for 4 hours with air as the gas phase. The contents of the vessels were then ground in a Potter-Elvehjem type homogenizer with a close-fitting teflon pestle. Three drops of glacial acetic acid were added to each homogenate and the mixtures deproteinized by allowing them to stand for 5 minutes in a boiling water bath. After centrifuging, the pH of each supernatant was adjusted to 9-10 with

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dilute NaOH solution and extracted twice with 10 ml portions of ethyl acetate. The extracts were combined. An adult rat was killed by decapitation, its liver dissected out, and a 50% liver homogenate prepared by grinding the tissue in 0.14 M KCl. To each of two 5 ml portions of the homogenate were added the following, all except the buffer having been previously neutralized: 400 μmoles of sodium acetate, 20 μmoles of sodium citrate, 10 μmoles of cysteine hydrochloride, 5 μmoles of coenzyme A, 10 μmoles of adenosine triphosphate, and 2.5 ml of tris (hydroxymethyl) aminomethane buffer at pH 8.0. To one of the vessels 100 μmoles of neutralized hydralazine hydrochloride were added and to the other an equal volume of water. The final volume of the mixtures was 12.5 ml in both cases. After a 2-hour incubation period, under the conditions described above, the mixtures were deproteinized and the supernatants extracted as above. *In vivo experiment.* A female guinea pig weighing 1.1 kg was injected subcutaneously with 10 mg of hydralazine hydrochloride and placed in a metabolism cage for urine collection. After an 8.5-hour interval, another 10-mg dose of hydralazine hydrochloride was administered. Twenty-six hours after the first injection, the collected urine was adjusted to a pH of 9-10 and extracted as the tissue supernatants above. A similar collection of control urine had been made previously. *Synthesis of acetylhydralazine.* One gram of hydralazine · HCl was dissolved in 10 ml of water and the solution made basic with dilute KOH. It was extracted twice with 10-ml portions of chloroform and the chloroform solution dried over anhydrous Na_2SO_4. The chloroform was removed by evaporating the solution to dryness in a round-bottom flask. The residue was taken up in 30 ml of benzene. Twenty milliliters of pyridine were added followed by 12 ml of acetic anhydride. This mixture was refluxed for 6 hours. After evaporation to dryness *in vacuo* at 100°, a brown, oily residue remained. This was dissolved in 95% ethanol, and water was added to incipient cloudiness. After standing in the cold for a short time, crystals formed. These were collected by centrifugation, recrystallized twice from ethanol-water mixtures, and once from 95% ethanol. After air-drying, the white crystals melted sharply at 178°. Microanalysis[†]: C, 59.41%; H, 5.11%; N, 27.66%: Calc'd: C, 59.41%; H, 4.95%; N, 27.72%. *Chromatography.* All paper chromatography was performed on strips 1″ in width of Whatman #1 filter paper by the ascending technic.

Results. When samples of ethyl acetate extracts of liver incubation mixtures or guinea pig urine were chromatographed in a number of solvents, brilliant white fluorescent zones were observed on strips under ultraviolet lamp in extracts from incubation mixtures containing hydralazine and from urine from the guinea pig given hydralazine. No such zone was found on strips prepared from control extracts. That this material was not unchanged hydralazine was shown by comparing chromatograms of hydralazine. Hydralazine does not fluoresce in the ultraviolet, but exhibits a dark brown absorbing zone. When chromatograms containing hydralazine were sprayed with a 0.1% alcoholic solution of picryl chloride and then exposed to ammonia vapor, a dark red-brown zone appeared. No such zone was apparent on chromatograms of the ethyl acetate extracts. Hydralazine showed different R_f values from those given by the white fluorescent material.

Extracts gave a chromatographic zone identical to that of synthetic acetyl hydralazine. Table I shows R_f values in a number of solvent systems of synthetic acetyl hydralazine and the zones obtained from either the guinea pig urine, the pigeon liver slices, or the fortified rat liver homogenate.

The white fluorescent material formed by pigeon liver from hydralazine and that present in guinea pig urine after administration of hydralazine was purified by successive paper chromatography in 22% isopropyl alcohol, water, n-butanol: 0.6 N NH_4OH (6:1), and 10 N acetic acid. After each chromatographic separation, the white fluorescent zones were cut from the strip, taking care to take only the most dense section and discarding the leading and trailing edges. The excised zone was cut into small pieces and eluted with 95%

[†] Schwarzkopf Microanalytical Lab., Woodside, N. Y.

ethanol. The ethanol extract was then used for the succeeding chromatograms. Fig. 1 shows the absorption spectra of the materials and of authentic acetyl hydralazine as determined on the Beckman DU spectrophotometer. The extract from the rat liver homogenate was carried through the same purification procedure; however it still exhibited appreciable absorption below 230 mμ. But in the range 240-250 mμ it gave an identical spectrum.

In none of the extracts was free hydralazine detectable. When the extracts were evaporated to dryness, taken up in 1 N HCl and allowed to hydrolyze on the steam bath for an hour, it was possible to demonstrate free hydralazine.

TABLE I. R_f Values of Acetyl Hydralazine and White Fluorescent Zone from Ethyl Acetate Extracts.

		White fluorescent zone from		
Solvent	Acetyl hydralazine	Guinea pig urine	Pigeon liver	Rat liver
22% isopropyl alcohol	.74	.72	.73	.72
60% *idem*	.85	.86	.84	
Water	.64	.62	.59	.60
2.5 N acetic acid	.81	.81	.82	
10 N *idem*	.86	.85	.89	.89
n-Butanol: 0.6 N NH_4OH (6:1)	.90	.88	.88	.89
20% $CHCl_3$: 80% n-butanol (water saturated)	.00	.00	.00	.00

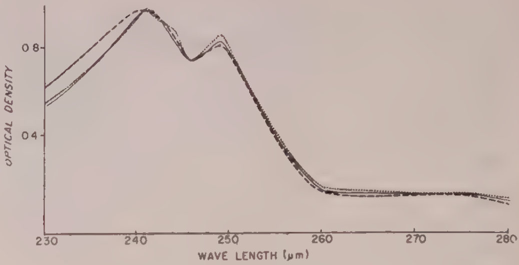

FIG. 1. Ultraviolet absorption spectra of authentic acetyl hydralazine (solid line), white fluorescent material from pigeon liver (dotted line), and guinea pig urine (dashed line).

From the above experiments, and from our previous(1) observations that for appreciable hydralazine disappearance from tissue extracts all the components of the metabolic acetylation system must be present, it seems evident that acetyl hydralazine is a major metabolic product of hydralazine.

Summary. 1-Acetyl-2-phthalazyl hydrazine (acetylhydralazine) has been identified as a metabolic product of hydralazine in the rat, guinea pig, and pigeon.

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Action of Biogenic Amines, Amine Oxidase Inhibitors, and Other Agents on Chromatophores of Squid, *Loligo pealii*.* (24658)

WILLIAM ROSENBLUM† AND BENJAMIN W. ZWEIFACH‡
Dept. of Pathology, N. Y. University-Bellevue Medical Center and
Marine Biological Lab., Woods Hole, Mass.

Regulation of smooth muscle-chromatophore system in the squid involves neurohumoral mechanisms which bear certain points of resemblance to smooth muscle systems in higher forms. Chromatophores of cephalopod molluscs have been extensively studied since the original work of Phisalix(1), but primarily for their own intrinsic value. Particular attention has been given to central neural controls. The purpose of the present experiments was to study local control of smooth muscle-chromatophore system in the squid, *Loligo pealii*. Particularly the action on this system, of biogenic amines known to be effec-

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† U.S.P.H.S. Summer Research Fellow.

‡ Established Investigator, Am. Heart Assn.

tive in higher forms as mediators of smooth muscle reactivity. Components of chromatophore system have the advantage of being easily accessible to treatment and observation in living, non-narcotized animals. The data clearly indicate that work with this preparation may shed light on problems of smooth muscle function in general.

Material and methods. Chromatophores of cephalopod molluscs(2,3) are pigment containing cells controlled by smooth muscles, which in turn are under the influence of animal's nervous system. When smooth muscle fibers contract, the cells expand and the animal darkens. Relaxation of muscle allows the cells to return to their initial, tiny, spherical shape, and the animal acquires a blanched appearance. *Loligo* has red, yellow, and brown chromatophores, the latter predominating(3). The skin is attached to the underlying mantel muscle by a dense adhesive layer. Injections made under the skin actually deposit the fluid between this adhesive layer and the mantel muscle. The injected material forms a circumscribed bleb which remains localized for a considerable time. Although the chromatophore bearing layer alone has been referred to as "skin," we shall refer to such injections as "subcutaneous." Sereni(4) has shown that chromatophores of cephalopods are controlled by separate excitatory and inhibitory centers in the ganglia in the head of the animal. These centers were found to exercise their control *via* nerves to the skin. Bozler(3) brought forth evidence for double innervation of muscle fibers, one innervation being excitatory (tetanic) and the other inhibiting tonus. He also presents microscopic evidence(5) that muscle fibers contain 2 types of fibrils. He assigns to one set of fibrils the function of ordinary contraction, and to the other the function of maintaining the fiber in a protracted contraction or tonus. In the present experiments, the squid were placed in confining glass bowl, slightly smaller than their fully extended length. The bowl was filled with sea water to a depth sufficient for animal to breathe, but exposing its convex dorsal surface to the air. The temperature had previously been dropped to about 10°C by placing the bowl in an ice bath. While experiments progressed, the water usually cooled further, to a minimum of about 5°C. Under these conditions, chromatophores of the squid generally dilate of their own accord, a condition essential for study of constricting substances. Test substances were dissolved in filtered sea water. The factor of pH will be discussed later. Four to 6 injections were administered subcutaneously to local areas along one side of exposed dorsum of animal, using 1 ml syringe and 24 gauge needle. A total volume of 0.2 ml was injected at each spot. Control injections of filtered sea water were made at locations symmetrical with those of experimental spots. Serotonin, as creatinine sulfate, was obtained from Sandoz Pharmaceutical Co. Iproniazid and Ro-5-0700 were kindly supplied by Hoffmann-La Roche Inc. and PIH by Lakeside Labs.

Results. Table I indicates substances injected into the squid, dose range used, and effects of injection. It should be kept in mind that closure of chromatophores represents relaxation of muscle and opening of chromatophore contraction of the muscle.

In vivo. A. *Agents which can open chromatophores.* Since it is well known that electrical stimulation of the skin, and acetylcholine (Ach) topically applied(3,4,5) cause chromatophores to open, these agents were used to test reactivity of the preparation. A 3 volt current with duration of 5 milliseconds, and with frequency of 60/sec. kept chromatophores of an area approximately one inch in diameter in open state during period of stimulation. The acetylcholine effect was most striking on a blanched squid, blanching being best obtained by cutting off the head of the squid. Injections should follow rapidly thereafter. Sea water alone causes a mild dilation in 27 out of 57 cases. The percentage of acetylcholine injections resulting in dilation is, however, much greater (48 out of 64 cases). In addition, dilation was considerably more intense with Ach than with sea water. The data are statistically significant, well within the 1% level of confidence when chi square test of significance is applied, using results from control injections as the expected results for the experimental series. Acetylcholine was effective in doses of 0.2 μg/ml or higher. These doses are at least 1000 times greater than values reported by other investigators. The different results may be accounted for by factors such as makeup of diluent medium, method of application, and particularly order differences. It may be significant that this order of cephalopod (especially the species used here) is unusually sensitive to trauma as contrasted with the octopus.

B. *Agents which close chromatophores.* (1) *Amines.* Kahr(6) reported that in the octopus, topical application of 5-hydroxytryptamine (serotonin) closed the chromatophores. This amine is known to have varied effects upon nerve-smooth muscle systems in general (7). In *Loligo,* serotonin causes blanching of skin when administered subcutaneously. However, the effective dose range was approximately 1000 times greater than that reported by Kahr(6). Sereni(4) reported that tyramine, administered to the octopus intravenously, caused opening of chromatophores, presumably by an effect on ganglionic centers controlling chromatophore activity. In our experiments, tyramine HCl, when injected subcutaneously, closes the chromatophores, an action diametrically opposite to its reported central effect. Tryptamine is reported by Woolley and Shaw(8) to have a serotonin-like effect in other systems. This was substantiated in the present preparation where it was found to close the chromatophores.

All of the active constricting agents (amines, inhibitors, indoles, etc.), except serotonin, cause the skin site to blanch to a white or near white color, in contrast to surrounding brown area. Although in general the area of blanching decreases in size with lower doses, minimal effective doses occasionally cause disproportionately large, pale spots. Occasionally, several minutes after constriction, scattered chromatophores open in the otherwise constricted area.

The responses to serotonin seemed qualitatively different from the others, in that the areas were uniformly pale but not white. The same response was obtained irrespective of whether the dose was 20 μg/ml or 2000 μg/ml of serotonin. Other agents cause skin areas to become white, due to closure of all chromatophores in the area. Such a reaction develops only occasionally with serotonin. More commonly, after serotonin, the red and brown chromatophores remained open, but the spaces between them paled considerably, losing their normal, yellowish cast. This yellow color is due to yellow chromatophores which are difficult to see as distinct entities in *Loligo,* since they are quite small. It is probable that the characteristic pallor following serotonin treatment, is due to a constriction chiefly of the yellow chromatophores.

(2) *Amine oxidase inhibitors.* The above experiments suggest that an amine might be the local mediator affecting closure of chromatophores. Assuming that such an amine was continually being destroyed by an amine oxidase, administration of an amine oxidase inhibitor should bring about the same effect as addition of the amine. Three inhibitors of monamine oxidase were used: PIH, iproniazid, and Ro-5-0700. All 3, as anticipated, cause chromatophores to close down. As can be seen in Table I, iproniazid was the least effective in this respect. This corresponds with the reported monamine oxidase inhibitory activities of these 3 compounds(9).

(3) *Indoles.* In addition to being an amine, serotonin contains an indole ring. LSD 25 is one of the indoles extensively studied with respect to serotonin-mediated systems. The brom derivative of this compound was tried by Kahr(6) on octopus chromatophores and he states that " . . . brom LSD opposes the effect of serotonin . . ." We found in the squid that LSD 25 produces the same effect as serotonin—*i.e.,* it closes chromatophores. However, LSD 25, although active, required much higher concentrations than serotonin.

(4) *Competitors for choline esterase.* Eserine sulfate was injected and had an effect only in large doses. Under such circumstances, instead of producing an Ach-like effect it closes the chromatophores.

(5) *Autonomic blockers.* Since amines and Ach are both believed to be important mediators of the autonomic nervous system in mammals, it was of interest to observe the effects of an autonomic blocking agent, chlorpromazine, on squid chromatophores. The drug closed the chromatophores.

TABLE I.

Agent	No. of exp.	Dose range* (μg/ml)	Effect
In vivo			
Electrical stimulation	20	5 volts	opens
Cholinergic			
Acetylcholine chloride	64	.2–20	"
Competitors for acetylcholine esterase			
Eserine sulfate	33	1000–2000	closes
Autonomic blockers			
Chlorpromazine	17	250	"
Nerve blockers			
Procaine HCl	8	1%–4%	"
Monamines			
5-OH-tryptamine (serotonin)	40	.2–2000	"
Tryptamine HCl	21	200–2000	"
Tyramine HCl	20	2500–4500	"
Catechol amines			
Epinephrine	10	100	*none*
Norepinephrine	23	100–200	"
Inhibitors of monamine oxidase			
1-isopropyl-2-isonicotinyl hydrazine (iproniazid)	36	1000–2000	closes
para-isopropyl hydrazine (PIH)	26	200–4500	"
2-benzyl-1-picolinyl hydrazine (Ro-5-0700)†	20	600	"
Amino acids			
L-lysine	6	2000	*none*
L-alanine	8	4000	"
Serotonin precursor			
5-OH-tryptophan	15	20–2000	"
Indoles			
LSD 25	15	100–2000	closes
Diamines			
Histamine dihydrochloride	5	.0002–20	*none*
In vitro			
Electrical stimulation	20	5 volts	opens
Acetylcholine	10	20	*none*
Histamine	6	20	"
Serotonin	15	.2–20	closes

* Low end of range represents dose of minimal effectiveness. Only 0.2 ml is inj. in single location.

† Dissolved in propylene glycol and administered as one part propylene glycol to 9 parts sea water.

(6) *Miscellaneous biogenic substances.* The amines mentioned above are all monamines, and the inhibitors were inhibitors of monamine oxidase. Adrenaline and noradrenaline are monamines with no effect, the former in doses up to 100 μg/ml. A diamine, histamine dihydrochloride, was tested in doses similar to those of the active compounds, without any effect on the chromatophores. A precursor of serotonin, 5-hydroxy-tryptophan(7) was also administered in comparable dose ranges and had no effect. Two monamines, L-lysine and L-alanine, not known to affect nerve-muscle preparations, were used to rule out a non-specific property of amines in general, or osmotic phenomena, and were without effect on the chromatophore system.

(7) *Interaction of oppositely acting agents on chromatophores.* Chromatophores opened by acetylcholine could be closed by subsequent subcutaneous administration of serotonin. The best results were obtained when equivalent doses of both agents were used. On the other hand, it was found that an electric stimulus of 5 volts, with duration of 5 msec. and frequency of 60/sec, failed to open chromatophores that had been closed with the above agents. The only possible exception to this finding occurs in serotonin-treated areas. As previously indicated, serotonin affected chiefly the yellow color. The chromatophores in such areas dilated upon electrical stimulation, to the extent that spaces between them disappeared. The resulting color is a rich brown, presumably due to combined effect of open red and brown chromatophores. This circumstance obscures any yellow color that might actually reappear upon such stimulation. To interpret the failure of treated areas to respond to electrical stimulation, it was necessary to determine whether electric stimulation normally dilates chromatophores because of an effect on muscle fibers directly, or because of stimulation of peripheral nerves. Bozler(3) reported that the effects of such stimulation are due to excitation of peripheral nerves. We attempted to corroborate this idea by using procaine, an agent known to block nervous conduction. Procaine HCl (1-4%) was injected into the skin in the usual manner and caused chromatophores to close. The chromatophores were then unreactive to electric stimulation.

(8) *Effects of pH.* Inasmuch as most substances used in our study were acidic in nature, it was necessary to rule out the effects of

pH *per se* on the reactions observed. The pH of sea water varies from 7.6 to 7.8. The different substances were tested, not only at pH they naturally assumed in sea water, but also after pH was brought to that of sea water (7.6-7.8), by adding small amounts of 0.1 *N* NaOH. The results at pH 7.6-7.8 were the same as those at lower pH values. The effects of pH alone were tested by injecting sea water acidified with HCl. Chromatophores are not sensitive to pH changes over a wide range(5-8). Lowering the pH on the acid side as far as 4, has no visible effect. Below pH 4, injections begin to cause constriction though this is not consistent until a pH of 3 is reached. These observations agree with those of Hill(10) who measured photoelectrically rapidity of response to a dilating electric stimulus. He showed no slowing of muscular response until pH was lowered below 5.

Chlorpromazine, LSD 25, or iproniazid could not be neutralized by this method without precipitate formation. Chlorpromazine solutions were at an unadjusted pH of 6.7 and the LSD was at an unadjusted pH of 6.5. Since this range of pH by itself had no effect, and since raising the pH of other agents did not alter their effects, we believe that the responses to LSD 25 and to chlorpromazine are probably not pH-dependent.

Injections of HCl ranging from pH 7 to 4 had no effect on chromatophores. Many of the active chemicals were administered as hydrochloride salts and therefore lowered the pH of seawater. Only one (iproniazid) lowered it below 6. Iproniazid lowered the pH to 3.5. Although sea water at pH 3.5 constricts chromatophores, iproniazid constricted them in 33 out of 36 occasions, whereas acidified sea water was positive in only 5 out of 32 occasions. Whenever possible, solutions of active substances were also adjusted back to pH of sea water by adding small amounts of .1 *N* NaOH. In no case did the findings differ from those made with solutions whose pH had not been adjusted.

In vitro. Experiments were also conducted with skin, excised from back of squid, and with pieces of tentacle. When lightly colored pieces of skin were stimulated electrically, the chromatophores opened. Pieces of skin were also placed in solutions of either acetylcholine, serotonin, or histamine. Both light and dark pieces were used. Only serotonin has an effect, lightening the dark tissue. The effective dose range was the same as for *in vivo* work. Kahr(6) reported findings in isolated skin similar to those above for serotonin and histamine, but in contrast to our study, he obtained dilation of octopus chromatophores with acetylcholine.

Discussion. The data on amines and amine oxidase inhibitors suggest that chromatophore control at the local level is achieved by local production or release of an amine, and its continual destruction by amine oxidase. Such an amine would relax smooth muscle and constrict the chromatophore. Another agent, such as acetylcholine, would also be produced locally and expand the chromatophore by contracting the muscle elements. The balance between these opposing agents could be upset by any one of several contingencies, adding an active amine, an amine oxidase inhibitor, acetylcholine, or autonomic blocking agent.

What are the agents concerned at the level of regulation? Of amines investigated, only serotonin acted in a dose range low enough to make it a reasonable possibility. It is of interest that Florey and Florey(11) found both serotonin and acetylcholine in the stellate ganglion of the squid, *Sepia*. Other adrenergic mediators, epinephrine, and norepinephrine, seem to have no local influence, although Sereni(4) found that systemic injections produce an expansion of chromatophores apparently through stimulation of the central ganglia. We must not overlook the possibility that permeability factors are of major importance here. Agents relatively ineffective by subcutaneous route may be much more effective by other routes.

The evidence obtained with amine oxidase inhibitors lend further support for local presence of an amine serving to relax muscle and thereby as an agent closing the chromatophores. Blaschko and Himms(12) have reported presence of monamine oxidase in different tissues of 2 species of squid. Apparently, diamines such as histamine are not concerned with funtcional responses of this nerve-muscle unit.

LSD 25 contains an indole nucleus, as does serotonin. It is known(13) to oppose serotonin in some systems and to mimic its action in others. Some biological properties of serotonin may be due to its indole nucleus, rather than its amine configuration. This would explain why another indole containing compound, LSD 25, mimics its action in the chromatophore system. The fact that minimal effective dose of LSD 25 is much higher than that for serotonin points to the highly specific nature of the receptor system in the chromatophore.

In our experiments, threshold concentration of serotonin was 1000 times greater than that reported by Kahr(6) for the octopus *Eledone*. He also reports in this cephalopod mollusc that brom LSD opposes the action of serotonin. However, as Page(7) indicates actions of LSD and its brom derivative are not always the same. The discrepancy in serotonin threshold may be due to differences in the order of mollusc used, or in method of application of the drug. The apparent differences in effectiveness of serotonin, as compared to other amines, may be due to 2 or more types of receptors. Woolley and Shaw(8), for example, have described different receptors for tryptamine and serotonin in the carotid artery of the dog. Sereni(4) showed that certain amines are carried by blood of the octopus and apparently act on ganglionic centers controlling chromatophores. Of the agents used by Sereni, tyramine, in the present study, was also found active locally. When injected intravenously, tyramine caused opening of chromatophores, while we found that its local administration causes contraction. Sereni felt that tyramine acted on ganglionic centers controlling chromatophores. He also found that acetylcholine, though causing expansion of chromatophores when locally administered, causes chromatophores to close when administered intravenously. Again he assumes intravenously administered compound to act upon ganglionic centers. The dual behavior of a single compound is analogous to the situation in mammals, where agents such as acetylcholine may have a dilating effect on end organs, when they come in contact with peripheral receptors, such as myoneural junctions, but exert a constricting effect when they contact more central receptors, such as synapse between pre- and post-ganglionic fibers. It is also possible, if, as Bozler(5) reports, chromatophore muscles are doubly innervated, that locally active amines may act preferentially on one or the other set of nerves.

A. Szent-Gyorgyi(14) has recently emphasized that substances able to transfer electrons may be of prime importance in processes leading to or synonymous with muscle contraction. Isenberg and Szent-Gyorgyi(15) have shown that tryptophan, serotonin, tryptamine, and LSD donate an electron to riboflavin and combine with it to form a stable free radical complex. It is of interest that 3 of the agents (serotonin, tryptamine, and LSD) relax smooth muscle in the squid chromatophore system. With the help of Dr. Isenberg, we have shown that PIH, tyramine, and eserine also form a red solution with riboflavin. In terms of basic mechanism involved in smooth muscle reactions, it is therefore suggested that some substances which relax the musculature of chromatophores in *Loligo* may act by virtue of ability to donate electrons to other molecules.

It has been assumed that substances interacting with enzyme systems (*e.g.*, eserine and PIH) have a demonstrable biologic effect because of this particular interaction. The present data suggest that the effect may be due to some other physico-chemical property, such as ability to donate electrons.

Summary and conclusions. (1) The multicellular system that controls chromatophores of *Loligo pealii* has been a useful tool for study of smooth muscle function in non-narcotized living animals. (2) Acetylcholine opened chromatophores. The following compounds closed them: Monamine-serotonin, tryptamine HCl, tyramine HCl; indoles-LSD 25; monamine oxidase inhibitors-iproniazid, PIH, Ro-5-0700; autonomic blockers-chlorpromazine; competitors for choline esterase-eserine sulfate. (3) Diamine, histamine dihydrochloride, had no effect. Other monamines, L-lysine and L-alanine, had no effect in comparable dosage. Catechol amines epinephrine and norepinephrine were ineffective. A precursor of serotonin, 5-hydroxytryptophan, had no effect. (4) Closure of chromatophores by an active substance was not reversible by electrical stimulation. (5) Attention is called to the fact that constricting agents all share a common property, that of resonance, and that a number of them are capable of electron donation. (6) Results obtained are consonant with theory that chromatophore control is achieved by an amine-amine oxidase system (closure), opposed by an acetylcholine-choline esterase system (expansion).

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Effect of Tolbutamide on Glucose and Nitrogen Metabolism in Totally Depancreatized Dog. (24659)

HENRY L. WILDBERGER AND HENRY T. RICKETTS
Dept. of Medicine, University of Chicago

The manner in which sulfonylureas lower blood sugar has not been fully elucidated. There is considerable evidence that they may stimulate release of insulin from the pancreas when that organ is present and functioning (1-6), and it is well established that the drugs are ineffective when pancreas is absent(1,7,8). We and others have demonstrated, however, that the sulfonylurea, tolbutamide, diminishes blood and urinary glucose in totally depancreatized dog maintained with suboptimal doses of insulin(9-13). Clearly, then, the pancreas is not essential for the action of this compound provided small amounts of insulin from other sources are available. The question now arises, whether, under these circumstances, tolbutamide potentiates the injected insulin or whether, alternatively, its action takes place primarily in tissues where insulin plays only a permissive role. In our experiments, to answer this question, changes in urinary nitrogen excretion of the inadequately treated diabetic animal were used as criterion of insulin potentiation.

Methods. Mongrel dogs approximately 10 kg were subjected to total pancreatectomy. Completeness of operation was verified at autopsy. The animals were kept in metabolism cages and given constant diet (Rival Dog Food) in 2 daily feedings with measured amounts of pancreatin. Acidified 24-hour specimens of urine were collected daily and combined in 3-day pools for later analysis. Feces were collected quantitatively over brief periods from 2 dogs. Total nitrogen was determined in food, pancreatin and feces after acid digestion, and also in urine, using steam distillation(14a) and titration with boric acid (14b). Pooled urine was analyzed quantitatively for glucose(15). Fasting blood sugar (16) was determined several times a week. Dogs were weighed periodically. Animals were maintained on constant suboptimal amount of regular insulin (2 to 6 units twice

TABLE I. Effect of Added Insulin Compared to Added Tolbutamide on Urinary Glucose and Nitrogen of Totally Depancreatized Dogs Inadequately Treated with Insulin.

		Mean blood sugar, mg %			Mean urine sugar, g/day			Mean urine N, g/day		
Dog	Wt, kg: Initial/terminal	All control periods	+ PZI	+ Tol.	All control periods	+ PZI	+ Tol.	All control periods	+ PZI	+ Tol.
28	12.0/7.0	302	254	217	32.1	6.8	5.9	9.2	7.8	9.3
32	7.8/5.4	314	265	221	34.6	13.0	24.2	6.1	5.6	5.9
19*	10.7/8.3	379	206		43.3	0.7		9.0	8.0	
36*	9.7/6.8	364		242	38.4		11.4	6.0		5.85
37*	8.8/6.3	302		246	38.9		25.8	9.2		9.6

* Died before some of planned observations could be completed.

Data for first 3 days of each period (control, PZI and tolbutamide) were excluded from calculations.

PZI = protamine zinc insulin, 2 units once daily. Tol. = tolbutamide, 0.25 g twice daily.

a day). No studies were performed sooner than 2 weeks after laparotomy. The experimental design consisted of 5 periods of 11 to 27 days each: (1) initial period when animal received only constant small doses of regular insulin; (2) experimental period when either supplementary protamine zinc insulin 2 units once daily, or tolbutamide 0.25 g twice daily, was administered; (3) recovery period following withdrawal of supplementary drug; (4) second experimental period employing alternate supplementary drug; and (5) final recovery period.

Results. Unfortunately, some animals succumbed before all of planned observations could be completed. Premature death of several dogs seemed attributable to extensive bleeding accompanied by high prothrombin time after treatment with tolbutamide(13). Similar results have been found with carbutamide(17) and isopropylthiodiazole(18).

A significant fall in urinary nitrogen, synchronous with reduction of glucose levels, occurred when supplementary protamine zinc insulin (PZI) was given (Table I).* In contrast, tolbutamide produced no demonstrable decrease in urinary nitrogen. Differences in nitrogen loss with PZI as compared with tolbutamide were statistically significant ($p < .01$).

Nitrogen balance, determined roughly during control observations for two 3-day periods in Dog 32 and for one 3-day period in Dog 36, seemed to be at equilibrium or slightly positive (+0.89, +0.3 and –0.08 g/day respectively).

Discussion. Nitrogen sparing effect of insulin(19,20) is demonstrated best, although probably not only(21), when nitrogen balance is negative(22). In our experiments, marked weight loss of all animals (Table I), in most to the point of cachexia, renders it probable that overall nitrogen loss exceeded intake despite what seemed to be equilibrium in 2 dogs during brief periods of observation. The endogenous origin of at least some urinary nitrogen is proved by decrement that occurred when small additional amounts of insulin were given. Tolbutamide, on the other hand, under similar conditions and in doses sufficient to cause significant reduction in glucose levels, was without effect on nitrogen excretion.

These observations are incompatible with the notion that tolbutamide potentiates exogenous insulin, at least in the dog. They can best be explained by the assumption that the drug reduced glucose output from the liver, as shown by earlier work(23), under permissive action of injected insulin.

Summary. In totally depancreatized dogs maintained on a constant regimen with suboptimal amounts of insulin, additional insulin, but not tolbutamide, caused a decrease in urinary nitrogen along with the expected decline in glucose levels. The failure of tolbutamide to duplicate the action of insulin suggests that it does not potentiate the injected hormone.

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* One animal with only mild glycosuria during control periods failed to show a fall in urinary nitrogen on PZI and was therefore excluded.

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Concentration of Azoproteins in Yolk Sac Placenta of the Rat.[*] (24660)

VERGIL H. FERM, H. JAMES FREE,[†] AND DANA L. SHIRES[‡]
(Introduced by James G. Wilson)
Dept. of Anatomy, College of Medicine, University of Florida, Gainesville

The striking role of the rabbit yolk sac placenta in transmission of homologous gamma globulin from the maternal system into the embryo and the apparent non-permeability of this membrane to heterologous protein has been described by Brambell(1). Few attempts have been made to study the permeability of either the chorio-allantoic or the inverted yolk sac placentae of the rat to protein molecules although both homologous and heterologous protein compounds have been used extensively in various studies of maternal-fetal relationships in this animal. The present work is an attempt to study the fate of 2 different azoprotein molecules injected into the pregnant rat, specifically with respect to placental membranes at various stages of gestation.

Materials and methods. The azo dye, echt säure blau B, has been shown to be rapidly excreted in the bile and urine of animals injected with moderate amounts of this compound(2). It is not phagocytyzed by the reticulo-endothelial system and apparently does not combine with serum proteins *in vivo*. Echt säure blau B, an intensely blue azo dye, was diazotized and coupled to crystalline bovine serum albumin and rat serum albumin utilizing the technic described by Kruse and McMaster(2). Bovine serum albumin was obtained commercially and rat serum albumin was obtained by ammonium sulfate precipitation of pooled rat serum. After final dialysis and filtration, the bovine serum albumin-azo dye preparation (BSA-ESBB) contained 20 mg of protein and 1408 μg of dye/ml. The final rat serum albumin-azo dye preparation (RSA-ESBB) contained 30 mg of protein and 1270 μg of dye/ml. Four ml of the BSA-ESBB preparation and 5 ml of the RSA-ESBB preparation were injected intraperitoneally into young nulliparous Sherman rats weighing 250-280 g on days 7, 9, 11, 13, 15, 18, and 19 of pregnancy. Each animal received only one injection and was sacrificed 24 hours later at which time maternal cardiac blood was drawn. Specimens of maternal liver, spleen, mesenteric lymph node, and kidney were fixed in Carnoy's fluid, 10% formalin, and alcohol-formol-acetic acid. Placental membranes and fetuses were removed and fixed in an identical manner. Method of fixation was found not to affect the histological results, consequently tissues fixed in 10% formalin were sectioned at 10 μ, cleared in xylene, left unstained, and mounted with coverslips for histological localization of the colored azoprotein material. Some sections were stained with hemotoxylin and eosin for comparison. Control animals were divided into 2 groups. The first group received intraperitoneal injections containing 0.25% to 1.0% dye and 3.0% bovine serum albumin or rat serum albumin, mixed just prior to injection. For the second group of control animals. the azo dye was diazotized as in the first stage of the preparation of the azoprotein but tyrosine was substituted for the protein molecule. This solution was prepared to test the possibility of intermolecular coupling of dye molecules and to observe any effect that coupling might have on the histological results. Control animals received injections at corresponding stages of pregnancy, the maternal animals sacrificed at the same intervals, and the tissues treated in the same manner as the experimental animals. Maternal sera of the control and experimental groups were examined for gross blue coloration, precipitated with cold 10% trichloracetic acid (TCA) and centrifuged.

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[†] Lederle Medical Student Research Fellow.

[‡] N.I.H. Medical Student Research Fellow.

Results. In all control animals the maternal sera were free of any color at time of sacrifice. Some control animals, examined after only 5 hours exposure to the unbound dye-protein mixture, showed heavy dye concentration in the biliary tree of the liver, in the lumen of the small intestine and in the urinary bladder. No blue color could be seen in the maternal kidney, or within the fetuses or their placental membranes. In those control animals examined after 24 hours exposure to the control solutions, no evidence of any blue coloration could be found in maternal sera, maternal tissues or in embryonic tissues.

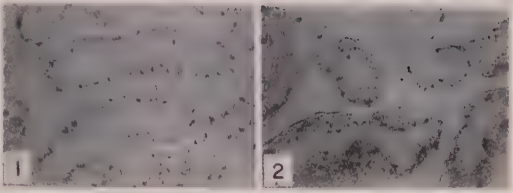

FIG. 1. Unstained cross-section of yolk sac placenta of a 19-day rat fetus, 24 hr after inj. of BSA-ESBB compound. Dark granules represent blue azoprotein material. 10 μ. 100×.

FIG. 2. Unstained cross-section of yolk sac placenta of a 19-day rat fetus, 24 hr after inj. of RSA-ESBB compound. Note the same general distribution of azoprotein material as in Fig. 1. 10 μ. 100×.

The maternal sera of the experimental animals receiving the BSA-ESBB preparation were quite blue at time of sacrifice and TCA precipitation yielded a definite blue precipitate with a clear supernatant. Histological examination of unstained sections revealed the blue azoprotein to be evenly distributed within the cells of the maternal renal tubules and in the mesenteric lymph nodes. Traces of the dye could also be found in maternal liver and spleen. Histological localization of the BSA-ESBB compound was apparent in all stages of the developing yolk sac placenta studied in this experimental group. As early as the eighth day of gestation the blue granules could be seen within the visceral layer of the yolk sac epithelium that lies next to the embryo proper. No azoprotein could be found in any other of the extra-embryonic tissues or in the embryo proper at this stage. At each succeeding stage studied, more obvious localization of the dye was apparent in the yolk sac endoderm and heavier concentrations appeared in the perinuclear cytoplasm of these cells (Fig. 1). At no stage of development was any of the BSA-ESBB compound found within the body of the embryo.

In those experimental animals injected with the RSA-ESBB preparation there was widespread distribution of the blue color at time of sacrifice. This color was evenly distributed throughout the body, coloring especially the subcutaneous tissues and fat deposits. The maternal liver, kidney and lymph nodes were grossly quite blue. There was detectable color in the maternal serum at time of sacrifice, but the coloration was not as intense as with the BSA-ESBB series. There was much more marked deposition of the blue azoprotein within the sinusoids of the maternal liver and within the lymph nodes of this experimental series than in the BSA-ESBB series. The renal tubules also showed concentration of blue pigment but in this series there was a tendency for it to be concentrated within the proximal part of the tubules. A strikingly similar concentration and distribution of the RSA-ESBB and the BSA-ESBB compounds was found within the visceral layer of the yolk sac placenta at all stages of gestation (Fig. 2). No histological evidence of the labeled rat serum albumin could be found within the embryo proper at any stage of gestation studied.

Discussion. The findings presented here offer direct visual evidence that the yolk sac placenta of the rat concentrates and apparently tends to block the transfer of both the BSA-ESBB and the RSA-ESBB preparations into the embryonic system at all stages of gestation studied. We cannot be certain that in labeling the rat serum albumin it has retained its species identity. It appears to behave differently in the maternal system than the BSA-ESBB compound, in that it is diffusely spread throughout the system, coloring subcutaneous tissues, mesenteries, and fat deposits as well as appearing in the renal tubules and yolk sac endoderm.

The problem of permeability of the placenta of rodents and closely related forms to protein molecules has been examined by various technics. Brambell(1) has shown that the permeability of the rabbit blastocyst to maternal serum albumin apparently ceases after the ninth day of gestation. Winkler(3), using radio-iodinated homologous serum albumin in the near-term rabbit, found some evidence of a slow transfer of the homologous albumin to the fetus after 24 hours of maternal exposure to this compound. Dancis(4) has reported that the guinea pig fetus is capable of synthesizing all of its own serum proteins except gamma globulin. He also presents data suggesting that there is some placental transfer of maternal serum proteins to the guinea pig fetus as measured by *in vivo* and *in vitro* radio-labeling of maternal serum. However. the main difficulty in interpreting data using radio-labeled proteins in studies of placental permeability is that there may be a breakdown and resynthesis of the protein-label in one or more of the placental tissues.

The limiting factor in this technic of tracing the fate of colored azoproteins is one of color dilution, and the technic does not permit final conclusions as to completeness of the placental block since it may be that very small amounts of protein get across the placental membrane into the embryo. The strength of the bond between the azo dye and the protein molecule, and the rapid elimination of any unlinked dye makes this technic a reliable one within the limitations mentioned.

In view of the above data, it would seem advisable to review the experimental data in which placental transfer of heterologous or homologous protein molecules has appeared to be an important factor in making definite conclusions. Such situations might exist in the experimental data accumulated in use of protein hormones in the study of the fetal endocrine system and, also, in the recently described phenomenon of actively acquired tolerance of the fetal system to antigenic material.

Summary. Bovine serum albumin and rat serum albumin were coupled to an azo dye, echt säure blau B to form colored azoproteins. Female rats at various stages of gestation were exposed 24 hours to these compounds and to control solutions. Both azoprotein molecules could be found within the kidney tubules, lymph nodes, maternal serum, and in all stages of the developing yolk sac placenta. No evidence of the dye in either case could be found in the embryonic tissues proper or within any of the other extra-embryonic membranes at any stage of gesta-

tion studied. The significance of this apparent impermeability of the rat placenta to these azoproteins is discussed.

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Proposed Method for Determining Biological Value of Protein in Ruminants. (24661)

J. W. Blair,* H. M. Page and E. S. Erwin (Introduced by A. R. Kemmerer)
Dept. of Animal Science, University of Arizona, Tucson

Little attention has been directed to the biological value of protein for ruminant animals because of the ability of the rumen microflora to synthesize amino acids. With the advent of nutritional interest in non-protein nitrogen, and more recently the interest in chemical stimulation of growth in ruminants, the need for an adequate simplified measure of biological value of protein has become important. Protein digestion and nitrogen retention have been used as methods for determining protein utilization by ruminants because no other satisfactory methods have been available. The Thomas-Mitchell method(1) for biological value determination, which has been generally used as the criterion in monogastric animals, has not proven satisfactory for ruminants. Because of the unique physiological functions of the ruminant digestive tract, accurate measurements of metabolic and endogenous nitrogen, required by the Thomas-Mitchell method, are not obtainable. However, estimates of endogenous and metabolic nitrogen calculated by Harris and Mitchell(2) from body weight and feed consumption, respectively, have been used in biological value determinations in ruminants.

In this investigation, alteration of blood and urinary nitrogen constituents from lambs was studied to develop a simplified method of: (a) predicting the biological value of protein, and (b) determining magnitude of stimulation from anabolic drugs.

TABLE I. Constituents of Experimental Rations, %.

Constituents	CSM	CSM-urea	Urea
Molasses beat pulp	36.5	36.5	36.5
Cotton-gin waste	35	35	35
Barley		12.05	24.1
Cottonseed meal	27.5	13.75	
Urea		1.7	3.4
Salt	1	1	1

Method. Twenty-six lambs were fed 3 rations (Table I) which varied only in source of nitrogen: (a) cottonseed meal, (b) 50% cottonseed meal : 50% urea, and (c) urea.

One-half of the lambs fed each ration was subcutaneously implanted at the base of the ear with 12 mg diethylstilbestrol. Rations were fed *ad libitum* for 10 days, followed by a 10-day period in which rations (17% crude protein) were fed in accord to 2.3% body weight. Blood samples, plus 24-hour urine and fecal samples were obtained from all lambs for analyses of nitrogen constituents. Apparent nitrogen (N) digestion was determined by means of the chromic oxide ratio technic. Thereafter, lambs were fed their respective rations *ad libitum*, and 45-day weight gains were calculated.

Observations. Illinois workers(3) reported that rations containing 11% protein equivalent satisfied the N requirements for maintenance and growth of lambs. Diets that contained 12% protein equivalent derived from urea N resulted in similar biological values

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* Present address: Valley National Bank, Phoenix, Ariz. Scientific Paper No. 459, Arizona Agric. Exp. Station, Tucson.

TABLE II. Summary of Results.

	Diethylstilbestrol			None		
	CSM*	CSM-urea	Urea	CSM	CSM-urea	Urea
Avg body wt, lb	94.7	94.8	90.7	96.3	98.1	83.8
Avg day gain, lb†	.506	.445	.402	.337	.218	.284
Urine constituents:						
Urine N, mg/ml	7.56	5.90	8.26	7.01	9.06	6.06
NH_3N, "	.34	1.26	.52	.26	.47	1.22
Creatinine, "	.648	.605	.753	.458	.543	.479
Creatinine N, "	.248	.226	.281	.171	.203	.179
Total N, "	11.37	11.10	13.40	8.99	11.28	11.06
Creatinine N, %	2.13	2.04	2.09	1.90	1.79	1.61
Apparent N digestibility, %	65.34	69.88	73.40	66.76	66.85	69.35
$\frac{\text{Creatinine N, \%}}{\text{Apparent N digested}}$	3.26 ± .51	2.92 ± .92	2.84 ± .24	2.84 ± .36	2.67 ± .20	2.32 ± .53

* CSM refers to cottonseed meal.

† Calculated for 45 days.

and N retention values in lambs as soybean meal N(3). However, when protein equivalent was increased to 17%, the N from protein sources proved superior to urea N(2,3). Johnson *et al.*(4) emphasized that the biological value of protein from various sources for lambs has been reported by many stations to approximate 60; however, these studies were conducted with lambs fed 10% to 12% crude protein. In the study reported here, 17% protein equivalent rations were fed in accord to body weight, with the attempt to emphasize the biological value difference between sources of dietary N and diethylstilbestrol.

The growth stimulation due to diethylstilbestrol administration (Table II) was similar to the results found elsewhere(5). Furthermore, growth response was similar to the results reported by Light, *et al.*(6), who found that the value of urea was increased when fed to diethylstilbestrol-treated lambs.

Neither creatinine nor non-protein N content of the blood (Table II) were indicative of chemical growth stimulation or source of dietary N. Also, urinary ammonia and urea were not consistently influenced by treatment (Table II). However, total N per ml of urine was increased progressively with increasing amounts of urea in the ration.

Whereas, creatinine concentration/ml of urine was variable, the percentage of total urinary N from creatinine (creatinine N%) was directly related to the expected N utilization and to the actual growth rate of lambs in the respective groups (Table II).

Twenty-four hour urine collections may not be indicative of the average urine excretion in lambs; the short-time duration of urine collection adds extreme variability, particularly if the lambs are not accustomed to metabolism stalls. However, urinary creatinine N% determination does not require the recording of urine volume.

Urinary creatinine N has been considered as endogenous N independent of quantity or quality of dietary protein(7). For the most part. urinary N, other than that from creatinine, must originate primarily from metabolic degradation of dietary amino acids or from the non-protein N that was absorbed via the rumen wall. The percent of total urinary N that originates from creatinine or urinary creatinine N% should be related to the comparative amount of absorbed N that was retained in the body. Further, this measurement deducts body size differences between animals by correcting for endogenous N. For the most part, creatinine N% is indicative of relative amount of absorbed N that is retained by the tissue. Although this measurement does not possess the theoretical exactness of the numerator in the Thomas-Mitchell formula [*N* intake—(fecal N – metabolic N) – (urinary N – endogenous *N*)], it is more applicable for ruminant studies. With dogs, others(8) have obtained a 0.946 and a 0.988 correlation between urinary creatinine N% and the Thomas-Mitchell method of biological value determination.

When urine was obtained from the lambs, 24-hour fecal samples were also collected. Apparent N digestion (Table II), calculated by the chromic oxide ratio technic, was used as the denominator for correcting the respective creatinine N% values. This measurement was related to relative growth response of the lambs. Although metabolic fecal N was not obtained, this calculation stimulates the Thomas-Mitchell formula for determination of biological value. Both methods determine the comparative amount of absorbed N that is retained by the animal. While the Thomas-Mitchell method is most accurate for monogastric animals, the method proposed here provides a simple and practical procedure for determining comparative protein anabolism or biological value of protein in ruminant animals. Further, this method should be of particular value in determining relative duration of influences of relative magnitude of effects of growth-stimulating drugs.

Summary. A method for predicting comparative values of nitrogen sources and anabolic protein stimulation by drugs in ruminants has been proposed. The method requires determination of urinary creatinine N% divided by the apparent N digested (expressed as a decimal) in animals with similar N intake per unit body weight.

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Rapid Method for Determination of Lipid Bound Radioiodine in Blood.

(24662)

Herbert D. Strauss and Harriet K. Richards (Introduced by J. O. Lampen)
Veterans Administration Hospital, Philadelphia, Pa.

Studies of absorption of radioactive fats, and fatty acids, labeled with I^{131} have recently been shown(1,2,3) to provide useful clinical information in certain disorders of the gastrointestinal tract. Although much on radioactive fat absorption has described measurements on fecal collections(4), more recent studies have shown that measurements of radioactivity in blood could also be correlated with clinical status. For example, in idiopathic steatorrhea, pancreatitis, and pancreatic insufficiency, blood levels of radioactivity have been shown to be below values obtained with normal individuals. The ease with which blood radioactivity measurements could be made compared to data obtained from fecal collections, has also provided impetus for evaluation of blood radioactivity. In addition to measuring total I^{131} radioactivity in blood, some investigators(1,2,5) have determined amount of radioactivity in protein-bound form. One report(5) indicated that certain disease states, such as hypercholesteremia, lipemia and coronary artery disease are associated with alteration of ratio of lipid-bound radioiodine to inorganic radioiodine. Interest in protein-bound I^{131} measurements in fat absorption with I^{131} labeled fats, has led us to seek a more rapid procedure than the trichloroacetic acid precipitation of protein. Anion exchange resins, useful in removal of inorganic I^{131} in protein-bound "conversion ratio" studies(6,7,8) might also be suitable for separation of lipoprotein I^{131} radioactivity. If this separation could be performed on whole blood without prior re-

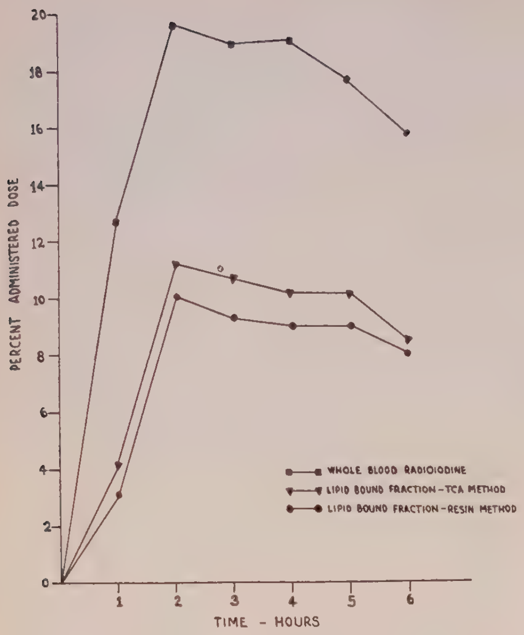

FIG. 1. Comparison of whole blood values and lipid-bound values done by TCA and Ion Exchange methods following a test dose of I^{131} labeled glyceryl trioleate.

moval of red blood cells, additional savings in time would result. Both of these objectives have been achieved through use of a new commercially available ion exchange kit.*

Materials and methods. A. *Trichloroacetic acid* (TCA) method. The procedure of Beres (1) and co-workers was followed with minor modification. B. *Ion exchange* method: Two ml of distilled water was added, with mixing, to 2 ml of heparinized radioactive whole blood in test tube. This mixture remained undisturbed 5 minutes to permit hemolysis of red blood cells. The red solution was now passed through the ion-exchange column. The column was then washed twice with 1 ml portions of isotonic saline to insure complete removal of protein-bound radioactivity from the column. These washes were added to original column eluate. All samples were counted in well-type scintillation counter. Since effluents from resin columns were not of equal volume and the well crystal is not linear for increasing volumes of fluid, it was necessary to make corrections in terms of counts observed versus volume of sample. A volume calibration curve was constructed for the well-counter so that all counting data could be corrected to the same volume. Eight subjects undergoing fat absorption studies with glyceryl trioleate were used. A total of 23 blood samples from these patients was used. Forty separate determinations were made with the TCA method, 14 of which were in duplicate. A regression analysis of the 23 pairs of percent TCA and percent resin determinations gives a correlation coefficient of 0.995 with a standard error of estimate of 0.44. Calculation of percent of administered dose present as lipid-bound radioiodine in whole blood is based on blood volume estimated as 75 ml blood/kg body weight. Therefore: % Lipid-bound radioiodine =

$$\frac{\text{Blood volume} \times \text{counts lipid-bound iodine/ml blood}}{\text{Counts original fat ingested}}$$

Results. These data show that both methods are reproducible to a high degree. In all cases, the resin method shows a slightly lower value than the TCA method. Nevertheless, similar curves may be obtained by serial determinations on the same subject, indicating that both technics are measuring the same entity (Fig. 1).

Summary. A method is reported for determination of lipid-bound radioiodine in blood by ion exchange resin technic. The method has been shown to be reproducible, accurate, rapid, and economical.

The authors wish to make acknowledgement to Mrs. Olha O. Holowecky for technical assistance, and Mrs. Winnie Strauss who prepared the graph.

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Incorporation of L-Carbamyl-C^{14}-Aspartate into Acid-Soluble Pyrimidine Nucleotides of Mouse Brain.* (24663)

WILLIAM T. MURAKAMI, DONALD W. VISSER, HAROLD E. PEARSON
Depts. of Biochemistry and Nutrition and Public Health, Univ. of Southern Calif. School of Medicine and Los Angeles County Hospital, Calif.

Our previous studies(1) showed that propagation of Theiler's GD VII strain of mouse encephalomyelitis virus in minced one-day-old mouse brain preparations results in increased uptake of P^{32} into ribonucleic acid phosphorus. Since it is known that acid-soluble nucleotides serve as precursors of cellular ribonucleic(2) and deoxyribonucleic acids(3), this study was undertaken to determine acid-soluble nucleotide constitution of noninfected and viral infected mouse brain and distribution of radioactivity in acid-soluble pyrimidine nucleotides and coenzymes following injection of a specific C^{14}-labeled pyrimidine nucleotide precursor, L-carbamyl-C^{14}-aspartate.

Methods. Synthesis of L-carbamyl-C^{14}-aspartate. L-carbamyl-C^{14}-aspartate was synthesized from urea-C^{14} (292 mg, specific activity 0.66 mc/mM), according to the method of Nyc and Mitchell(4). The alkaline reaction mixture was neutralized with concentrated HCl and the L-carbamyl-C^{14}-aspartate preparation was purified by separation on Dowex 1 (formate) X8 ion-exchange resin by the method of Lieberman and Kornberg (5). Based on the method of Koritz and Cohen(6) for determining carbamyl compounds, the specific activity of the final produce was 5.7×10^5 cpm/μmole. The calculated specific activity, based on urea, was 5.8×10^5 cpm/μmole. Although the L-carbamyl-C^{14}-aspartate solution was stored in deep-freeze, a chemical conversion to 5-carboxymethylhydantoin-C^{14} and to a smaller extent, dihydroorotate-C^{14} occurred. As much as 37% of total counts in the L-carbamyl-C^{14}-aspartate solution used for injection in Exp. 1 and 2 was present as 5-carboxymethylhydantoin. Chemical conversion was not detected when a neutralized L-carbamyl-C^{14}-aspartate solution was stored under similar conditions. *Injection of mice and preparation of acid-soluble fraction.* Mice, 5 to 6 weeks old, were injected intracranially in 6 sites with total of 0.024 ml L-carbamyl-C^{14}-aspartate solution (1×10^6 cpm/mouse). Mice were sacrificed 40 minutes after injection by cardiac exsanguination and brains were immediately dissected out and frozen by compression with dry ice. Frozen tissue was ground to a fine powder and homogenized with 2 volumes of ice-cold 0.6 N perchloric acid in tissue grinder, with a Teflon pestle. The homogenate was centrifuged and the protein pellet was extracted with 2 volumes of cold 0.2 N perchloric acid. Supernatant solutions from the 2 extractions were combined and perchloric acid was precipitated by addition of 5 N KOH to a phenol-red endpoint in an ice bath. After removal of $KClO_4$ by centrifugation, the perchloric acid-soluble fraction was chromatographed on a Dowex 1 (formate) X8 resin column. *Ion-exchange chromatography.* Purine and pyrimidine nucleotides present in perchloric acid-soluble extracts of mouse brain were separated by extended gradient elution chromatography system (modification of chromatographic system of Cohn(7) developed by Hurlbert *et al.* (8)). A more gradual formic acid gradient (mixer volume 1 liter, reservoir solution 2.3 N formic acid) was used for preliminary elution to get greater resolution of earlier

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* Aided by grant from Nat. Inst. of Health and Am. Cancer Society.

TABLE I. Incorporation of L-Carbamyl-C^{14}-Aspartate into Acid-Soluble Pyrimidine Nucleotides of Mouse Brain after 40 Min. Exposure.

	Exp. 1*		Exp. 2†		Exp. 3‡	
Nucleotide	Total c.p.m.	S.A.§	Total c.p.m.	S.A.	Total c.p.m.	S.A.
Carbamyl-aspartate	2×10^6		4.2×10^6		8.2×10^6	583,800
5-carboxymethyl-hydantoin	631,300		2.6×10^6		128,550	
Orotic acid	313,650	541,000	498,240	558,900	526,300	544,000
UMP	56,000	52,600	169,600	12,800	148,800	12,800
UDP	9,600	41,800	71,940	11,000	65,000	10,800
UTP			7,200	11,700	2,260	11,320
UDPG	2,600	26,000			7,200	9,450
UDPAG	4,750	12,000			10,750	4,460
CTP	560	6,000	330	1,500	240	1,300
CDP			580	1,170	360	1,100
CMP	680	2,400	1,360	930	3,210	900
CDP-ethanolamine					930	740

* 30 mice inj. with total 2.82×10^7 c.p.m. L-carbamyl-C^{14}-aspartate (S.A., 5.7×10^5).

† 100 mice inj. with total 1.17×10^8 c.p.m. Radioactive brain preparation diluted with extract 230 noninj. mouse brains.

‡ 90 mice inj. with total 9.08×10^7 c.p.m. Radioactive brain preparation diluted with extract 196 noninj. mouse brains.

§ Specific activity (S.A.) is count/min./μmole. Stand. dev. of counting rates were 2% or less.

peak components. *Analytical methods.* Isolated nucleotides were identified by absorption spectra in 0.01 N HCl, phosphorus analysis and in some instances by paper chromatography. Inorganic phosphate was determined by the method of Chen *et al.*(9). Total phosphate was determined after digestion with 6 meq sulfuric acid, labile phosphate following treatment of the sample with N H_2SO_4 for 15 minutes in a boiling water bath. Koritz and Cohen's(6) method for determination of carbamyl compounds was used for quantitative determination of carbamylaspartate and 5-carboxymethylhydantoin after quantitative conversion of the latter to carbamylaspartate by hydrolysis in N NaOH for 20 min at 100°. *C^{14} analysis.* Radioactivity of eluate samples was measured in 0.2 ml aliquots on aluminum planchets, 5.3 cm^2. Planchets were counted with an end-window counter (Model D-47, Nuclear-Chicago) and Nuclear Automatic Sample Changer. Radioactivity of purified nucleotide fractions was determined in duplicate, at infinite thinness with internal flow Q-gas counter (Model D-46, Nuclear-Chicago) on aluminum planchets.

Results. Nucleotide composition of adult Mouse Brain. The following purine and pyrimidine nucleotides were isolated and identified from mouse brain: AMP, ADP, ATP, GMP, GDP, CMP, CDP, CDP-ethanolamine, UMP, UDP, UTP, UDPAG and UDPG. In addition, a peak with A_{280}/A_{260} ratio corresponding to guanosine-containing compound and elution position characteristic of GTP was noted; this fraction was not further characterized.

Distribution of radioactivity. Distribution of radioactivity among perchloric acid-soluble components of mouse brain, 40 minutes after injection of L-carbamyl-C^{14}-aspartate into brains of 30 mice, is presented in Table I (Exp. 1). Several radioactive non-ultraviolet absorbing peaks were observed in addition to those of known pyrimidine nucleotides. Two of these were identified as 5-carboxymethylhydantoin and L-carbamylaspartate by carrier recrystallization and by ion-exchange chromatography following addition of nonradioactive material. Others were not identified. Specific activities of the recovered nucleotides are presented in Table I (Exp. 1). Specific activity of recovered orotic acid indicates little or no dilution by an endogenous pool. Labeling sequence of uridine nucleotides is similar to that in rat liver and in microorganisms(10). Specific activities of CMP and CTP are of particular interest since the higher level of C^{14}-incorporation into CTP indicates that uridine and cytidine nucleotide interconversion in mouse brain occurs at the triphosphate level by a reaction similar to that in cell-free extracts of *E. coli*(11). Much radioactivity was found in 5-carboxymethylhydantoin, which was later found to be a contaminant of the injected L-carbamylaspartate solution.

To obtain sufficient material for characterization of unidentified radioactive peaks, of CDP and UTP, 100 mice were injected with L-carbamyl-C^{14}-aspartate and sacrificed 40 minutes after injection. Acid-soluble components were increased by addition of acid extract of 230 non-injected brains. Specific activities of recovered pyrimidine nucleotides are given in Table I (Exp. 2). Specific activities of recovered pyrimidine compounds were about one-third those in Exp. 1, except for orotic acid. Since the specific activity of orotic acid was not changed by dilution with extract of noninjected brains, endogenous orotic acid level of mouse brain probably is very small. The results verify the conclusions from Exp. 1. Although more counts were recovered in radioactive nonultraviolet absorbing fractions than in the previous experiment, the amounts were insufficient for characterization.

To test the effect of 5-carboxymethylhydantoin-C^{14} on conversion of L-carbamyl-C^{14}-aspartate to pyrimidine nucleotides, 90 mice were injected with a total of 9.08×10^7 cpm of carbamylaspartate solution containing only 1% of the total radioactivity as contaminating 5-carboxymethylhydantoin. Animals were sacrificed 40 minutes after injection of isotopic material. Extract of radioactive brains was diluted with extract from brains of 196 noninjured animals. After injection of purified L-carbamyl-C^{14}-aspartate solution, all non-ultraviolet absorbing radioactive fractions except 5-carboxymethylhydantoin and carbamylaspartate were absent. Specific activities of pyrimidine nucleotides in this experiment (Table I, Exp. 3) are in good agreement with those from animals injected with mixture of 5-carboxymethylhydantoin and L-carbamylaspartate. This suggests that injected radioactive 5-carboxymethylhydantoin does not significantly affect conversion of carbamylaspartate to pyrimidine nucleotides. 5-carboxymethylhydantoin-C^{14} and L-carbamyl-C^{14} were recovered from acid-solubles in about same ratio as was present in the injected solution. This suggests that there was little or no conversion of L-carbamyl-C^{14}-aspartate to 5-carboxymethylhydantoin, *in vivo*.

Ability of mouse brain tissue to carry out the reverse reaction, conversion of 5-carboxymethylhydantoin to carbamylaspartate, was tested by injection of 36 mice with a total of 2.28×10^7 cpm purified 5-carboxymethylhydantoin-C^{14}. Animals were sacrificed 40 minutes later. Six radioactive fractions were recovered from perchloric acid-soluble fraction. Three of these were identified respectively as 5-carboxymethylhydantoin (2.4×10^6 cpm), carbamylaspartate (14,000 cpm) and UMP (1000 cpm). Remaining fractions possessed elution characteristics of non-ultraviolet absorbing radioactive peaks observed in Exp. 1 and 2. These results show that metabolic conversion of 5-carboxymethylhydantoin to carbamylaspartate does not occur to any great extent in mouse brain.

Virus infected mouse brain. Distribution of C^{14} in acid-soluble nucleotides of mouse brain was determined after injection of L-carbamyl-C^{14}-aspartate into mice previously infected with Theiler's GD VII strain of mouse encephalomyelitis virus. Forty-three mice were each injected intracerebrally with 0.03 ml virus (10^3 MLD_{50}). Virus-infected mice were injected intracranially with L-carbamyl-C^{14}-aspartate after first paralytic signs in the group, about 72 hours after injection of virus. Thirty-six mice were sacrificed 40 minutes later. Neutralized perchloric acid-soluble extract was made and chromatographed. Resultant chromatogram was almost identical with that for noninfected brain tissue. No significant differences were found in quantities of ultraviolet absorbing and radioactive materials of virus-infected and normal mouse brain. Specific activities of 3 radioactive pyrimidine nucleotides from virus-infected preparation, UMP-CMP and CDP were identical with those in noninfected brain.

Discussion. The acid-soluble nucleotide composition of mouse brain tissue is similar to that of other tissues(8,12,13). Specific activities of isolated pyrimidine nucleotides labeled from L-carbamyl-C^{14}-aspartate indicate that pyrimidine biosynthesis in mouse brain proceeds through a sequence of reactions which have been shown to occur in other tissues(10). Specific activity of recovered orotic acid suggests that there was very little dilution of the radioactivity of carbamylaspartate by unlabeled metabolic pools of dihydroorotate and orotate. Hurlbert and Potter(14) obtained evidence that the size of the metabolic pool of orotic acid in rat liver is very small. When the distribution of radioactivity of rat liver nucleotides was determined, following administration of orotate-C^{14}, practically all radioactivity could be accounted for as uridine nucleotides and no radioactive orotate, dihydroorotate or orotidylate were recovered. Thus it is probable that dihydroorotate and orotidylate which were not detected in the acid-soluble fraction from mouse brain, are present in very small amounts as intermediates of transient existence in mouse brain tissue. This assumption would account for the finding that carbamylaspartate is converted to orotate without dilution.

Incorporation of carbon-14 into all uridine nucleotides was more rapid than into cytidine nucleotides, thus fulfilling one criterion for a precursor-product relationship. The specific activity values of the uridine and cytidine nucleotides are in accord with the fact that the only known reaction for conversion of a uridine to a cytidine compound is that shown to occur at the nucleoside triphosphate level by Lieberman(11) with an extract of *E. coli.*

Ion-exchange chromatography of acid-soluble nucleotide fraction of mouse brains injected with stored solution of L-carbamyl-C^{14}-aspartate solution revealed presence of several radioactive non-ultraviolet absorbing peaks in addition to carbamylaspartate and 5-carboxymethylhydantoin. These unidentified compounds were not detected when pure L-carbamyl-C^{14}-aspartate was injected into the mice but did appear in each instance when 5-carboxymethylhydantoin-C^{14} was administered. This would imply that they are not normal intermediates of carbamylaspartate metabolism but may represent catabolic products of 5-carboxymethylhydantoin. The data also show little conversion of 5-carboxymethylhydantoin to carbamylaspartate; hence the former compound is not readily converted to pyrimidine nucleotide compounds.

From previous studies in this laboratory which showed a stimulatory effect of propagation of Theiler's GD VII virus on uptake of P^{32} into RNA of minced one-day-old mouse brain preparations(1), it is felt that the apparent similarity in distribution of the label of L-carbamyl-C^{14}-aspartate among the acid-soluble pyrimidine nucleotides of virus-infected and non-infected tissues does not reflect a lack of effect of virus propagation on acid-soluble pyrimidine metabolism of the tissue, *in vivo,* but rather serves to emphasize the need for a cellular system in which homogeneity of the cell population and infection of most of the cells by the virus can be more adequately controlled. Work is now in progress to develop such a system with mouse brain tumor tissue culture cells.

Summary. Acid-soluble pyrimidine nucleotides were separated by gradient elution ion-exchange chromatography after intracranial injection of L-carbamyl-C^{14}-aspartate into mice. Radioactivity was associated with orotic acid, UMP, UDP, UTP, UDPAG, UDPG, CTP, CDP, CMP and CDP-ethanolamine; this shows that brain tissue is capable of synthesizing these pyrimidine nucleotides from simple precursors. Specific activities of isolated pyrimidine nucleotides indicate that biosynthesis of pyrimidine nucleotides by brain tissue occurs in a manner similar to that in rat liver and bacterial extracts. Comparison of specific activities of CTP, CDP and CMP suggests that uridine to cytidine nucleotide conversion takes place between UTP and CTP in mouse brain, probably by a reaction similar to that shown by others in extracts of *E. coli.* Distribution of radioactivity was the same in brains of mice previously infected with Theiler's encephalomyelitis virus and in noninfected brains.

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Transfer of Experimental Panophthalmitis in Rats by Parabiosis.*† (24664)

E. F. Pfeiffer, K. Schöffling, G. Treser, I. Bachrach, W. Menk, E. Kurus, J. Otto and W. Sandritter (Introduced by J. Freund)
Depts. of Medicine, Ophthalmology and Pathology, Johann-Wolfgang-Goethe-University of Frankfurt, Germany

Transfer of experimental encephalomyelitis and glomerulonephritis by parabiosis was accomplished in rats(1,2). Transfer of glomerulonephritis has been demonstrated in both random bred and inbred strains of rats (3-5). The question arose, however, whether these transfer phenomena also held true for sensitization processes in other organs. In our experiments, allergic panophthalmitis has been produced by injecting rabbit-anti-rat-eye-serum into rats. The ophthalmitic animals were then joined with normal rats in parabiosis. Here, too, transfer of inflammation of the eye on the originally healthy partners has been achieved.

Methods. a) *Rabbit-anti-rat-eye-serum.* Frozen eyeballs of white rats of different breeds were triturated by the ballmill, diluted with saline to 20% suspension and injected 3 times a week for 2 months, and subsequently twice a week during 1 month, intraperitoneally into rabbits weighing 3-4 kg. Then the rabbits were bled from carotid arteries, blood collected under aseptic conditions, and antisera pooled and stored in 10 ml amounts in deepfreezer. b). *Cytotoxic panophthalmitis.* White rats of one strain (Stamm Zeilsheim) weighing 80-100 g, were injected with cytotoxic rabbit-anti-rat-eye-serum. Most animals were injected intravenously with single dose of 1-1, 8 ml of antiserum. Some rats also received that amount in 2 to 5 more daily injections. Starting 24 hours after first injection, the fundi of eyes were examined ophthalmoscopically every second day during experiment. The rats were sacrificed between 5th and 86th day after application of the antiserum. Whole eyes were fixed in 4% formalin, and sections of whole eye as well as of different tissues of eye were stained with H. + E. and PAS for microscopic examination. Sections of brain, heart, liver, spleen, and kidneys also were fixed in 4% formalin and stained with H. + E. Rats of the same strain, serving as controls, were untreated or injected with normal rabbit serum. Others were injected with 1 ml rabbit-anti-rat-kidney-serum intravenously. c) *Parabiosis.* As in former experiments(2) the parabiotic opera-

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TABLE I. Ophthalmoscopic and Histologic Signs of Panophthalmitis in 40 Rats Injected with Rabbit-Anti-Rat-Eye-Serum.

Group	No. of rats	Amt of antiserum	Occurrence of 1st ophthalm. findings (days after inj.)	Sacrifice (days after inj.)	Degree of ophthalmoscopic findings	Degree of histological findings
			Day			Degree
A	12	.7–1.8	2 × 1st 7 × 3rd 1 × 7th	2–21	2 × — 4 × + 1 × ++ 5 × +++	3 × 0 3 × 1st 2 × 2nd 2 × 3rd
B	12	1.0–3.0	1 × 1st 4 × 3rd 5 × 7th 1 × 9th	23–35	1 × — 2 × ++ 9 × +++	1 × 0 2 × 1st 4 × 2nd 5 × 3rd
C	10	1.0–2.0	3 × 1st 4 × 3rd 1 × 7th 1 × 9th 1 × 20th	36–59	2 × ++ 8 × +++	2 × 1st 5 × 2nd 3 × 3rd
D	6	1.5–2.0	2 × 1st 3 × 7th	69–86	1 × — 1 × + 4 × +++	1 × 0 2 × 1st 2 × 2nd 1 × 3rd

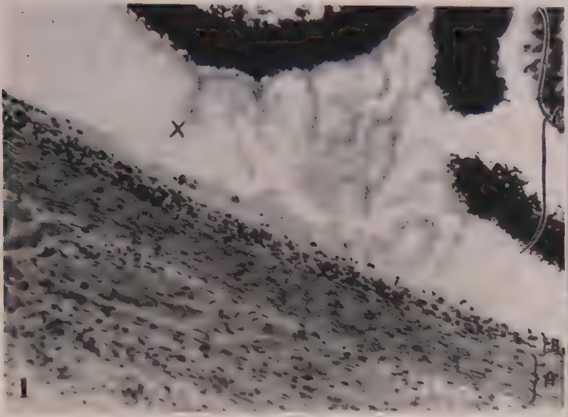
tion was performed by the method of Bunster and Meyer(6). By this technic, exchange of body fluids between parabiotic partners is based on capillary blood shunt established 3-4 days after operation. As in previous studies female rats have been used exclusively. Inbred Albino-Wistar rats (Stamm Koeln) and random bred white rats of one strain (Stamm Zeilsheim), in which ophthalmitic lesions have been recorded by ophthalmoscopic examination, were joined in parabiosis with healthy partners 8-43 days after the single or last injections of the cytotoxic rabbit-anti-serum. The animals were kept in parabiosis for 5-49 days and the same clinical and histologic examinations as described above for single animals were carried out in the parabionts. Parabiotic pairs without any injections were used as controls.

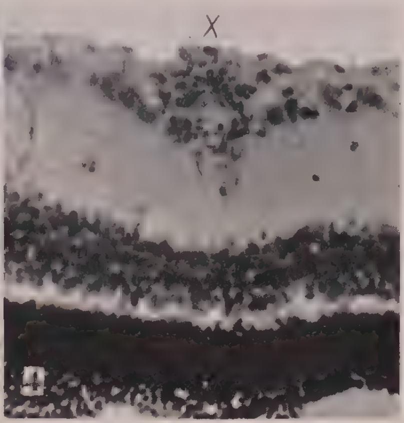
Results. Table I demonstrates the degree of clinical and pathological findings observed in 36 of 40 *single* rats, injected with rabbit antiserum. Clinical and histologic findings were divided in 4 degrees. The following symbols are used to indicate:

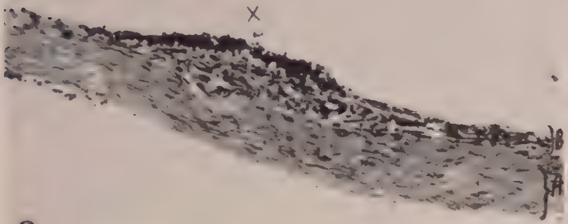
I. Ophthalmoscopic findings: — no pathologic findings; + hyperemia of fundus vessels, appearance of praecapillaries; ++ hyperemia of fundus vessels, appearance of praecapillaries, aneurysms, small bleedings from capillaries; +++ hyperemia of fundus vessels, appearance of praecapillaries, many aneurysms, perivascular sheath, large hemorrhages of retina, development of retroretinal exudate, sometimes followed by detachment of retina.

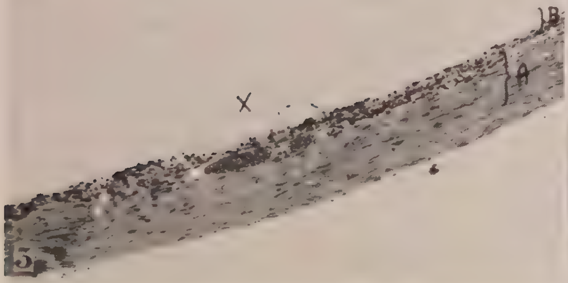
II. Histologic changes: 0 degree no pathologic changes. 1st degree hyperemia of iris, uvea, ciliary body, and retina. Vascularization of retina; 2nd degree hyperemia of iris, uvea, ciliary body, and retina. Vascularization of cornea, small infiltration of round and plasma cells in iris, cornea, and mostly in uvea; 3rd degree hyperemia of iris, uvea, ciliary body, and retina. Vascularization of cornea, compact infiltration of uvea, retina, and iris with round and plasma cells, occasionally eosinophiles. Edema of tissues of the eye, mostly of cornea. Retinal detachment, due to retroretinal exudation.

The first lesions were recorded by ophthalmoscopic examination within 24 hours after injection in 8 rats, within first 3 days in 15 others. They consisted of hyperemia of the retina and the chorioid tissue. Aneurysms and bleedings from capillaries of retina followed. Detachment of retina, due to retroretinal exudation, was observed in 11 animals. In one rat the lens became opaque, in 35 inflammatory changes were revealed by histo-









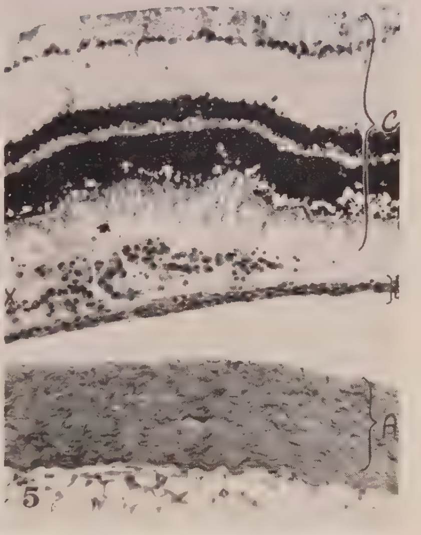


FIG. 1. Retroretinal exudation and secondary detachment. Intense exudation from densely infiltrated chorioidea resulted in retinal detachment. Rat eye stain: Hematox. + eosin. A = Sclera; B = Uvea; C = Retina; X = Exudate.

FIG. 2. Infiltration of round cells in chorioidea. At (X) dense concentration of small round cells. Chorioidea has taken on a cushion-like thickening due to edema. Rat eye stain: Hematox. + eosin. A = Sclera; B = Uvea.

FIG. 3. Perivascular infiltration of the chorioidea. (X) Infiltration of small round cells grouped around vessels in chorioidea. Rat eye stain: Hematox. + eosin. A = Sclera; B = Uvea.

FIG. 4. Perivascular infiltration of the retina. (X) Dense perivascular infiltration of round cells around a retinal vessel. Insignificant edema of retina. Rat eye stain: Hematox. + eosin.

FIG. 5. Infiltration of plasma cells. Massive immigration of plasmacellular elements from chorioidea into retroretinal space. At (X) densely concentrated plasma cells and retroretinal exudation. Secondary detachment in initial stage. Rat eye stain: Hematox. + eosin. A = Sclera; B = Uvea; C = Retina; X = Plasma cell infiltration.

logical examination. Fig. 1, section of eye, shows morphological lesions in one of these animals.

Twenty-eight rats, in which pathological findings were recorded by clinical examination after injection of antiserum ("primarily ophthalmitic animals") were joined in *parabiosis* with normal animals ("originally healthy partners") 8-43 days after injection. Three rats of the parabionts died within first 5 days after operation. In 21 of the remaining 25 pairs the ophthalmitis has been transferred on

TABLE II. Occurrence of Ophthalmitis (Recorded by Ophthalmoscope) in a Parabiotic Pair.

Days of exp.	Exp. procedures	Hyperemia of fundus vessels		Mikroaneurysms		Bleeding		Retroretinal exudate		Detachment of retina	
		P	O	P	O	P	O	P	O	P	O
1	Inj. of 2.0										
3	serum	+									
5		+		+							
7		+		+							
9		+		+							
11	Parabiosis	+		+							
13		+		+		+					
15		+	+	+		+					
17		+	+	+		+					
19		+	+	+	+	+					
21		+	+	+	+	+		+			
23		+	+	+	+	+	+	+			
25		+	+	+	+	+	+	+			
27		+	+	+	+	+	+	+			
29		+	+	+	+	+	+	+		+	
31		+	+	+	+	+	+	+		+	
33	Sacrifice	+	+	+	+	+	+	+	+	+	+
35		+	+	+	+	+	+	+	+	+	+

Histologic findings:

P = Primarily ophthalmitic animal—Compact infiltration of uvea with round cells, plasma cells and eosinophiles. Retroretinal exudate, detachment of the retina. General hyperemia respectively edema of the tissues.

O = Originally healthy partner—General hyperemia of uvea and retina. Infiltration of round cells in uvea and iris. Vessels in cornea. Detachment of retina.

the originally healthy partners.

Table II exemplifies in one pair time relations between injection of rabbit antiserum into the first animal, development of ophthalmitic lesions, recorded by ophthalmoscopic examination, parabiotic union and, eventually, occurrence of ophthalmitis in the originally healthy animal together with final histologic findings.

Table III summarizes the same relations in all parabiotic pairs joined in parabiosis at various intervals after injecting rabbit antiserum into the primarily ophthalmitic animal. Without relation to these different intervals, panophthalmitis occurred in originally healthy parabionts between 4th and 12th day after parabiotic union, and the 11th and 50th day after injection of antiserum into primarily ophthalmitic partners respectively. Histologic findings in the originally healthy parabionts were similar to those recorded in single animals injected with rabbit antiserum, and injected animals of the parabiotic group: Infiltration of round cells in uvea with edema in tissues (Fig. 2). Perivascular infiltration of round cells in chorioidea (Fig. 3), and in retina (Fig. 4), infiltration of plasmacells from uvea into the retroretinal space and beginning of retinal detachment (Fig. 5). No pathological findings were observed in sections of brain, heart, spleen, liver, and kidneys in either of these groups. Only occasionally small areas with infiltration of round cells were present in the injected animals in interstitial tissues of the liver. On the other hand, lesions of the eye were completely lacking in all untreated single animals, in 10 rats injected with normal rabbit serum and in 10 parabiotic controls. No signs of ophthalmitis were found in 10 rats injected with anti-rat-kidney-serum of rabbits. In several animals of this group, however, the well known signs of hypertensive diseases have been recorded in eyegrounds after development of albuminuria and hypertension.

Discussion. The experiments show that allergic panophthalmitis can be easily produced by injecting rabbit-anti-rat-eye-antibodies into rats. These observations are completely in accord with the findings of Golowin(7), who produced iridocyclitis by injection of antibodies against ciliary body ("cyclotoxins").

TABLE III. Incidence of Panophthalmitis in Parabiosis.

Group	Rat No.	Interval between inj. and institution of parabiosis (days)	Occurrence of first ophthalmoscopic changes in orig. healthy rats (days after parabiosis)	Degree of ophthalm. findings		Degree of hist. findings		Day of sacrifice	
				P	O	P	O	After inj.	After parabiosis
E	41	8	6	++	+++	1	3	26	19
	42	8	4	+++	+++	3	3	13	6
	43	8	—	+	0	1	0	14	7
	44	8	—	+	0	1	0	14	7
	45	8	6	+	++	1	2	20	13
	46	8	4	+++	+++	3	2	56	49
	47	8	6	+++	+++	2	1	23	16
	48	9	—	+	0	1	0	9	1
	49	9	8	+++	++	3	3	59	51
	50	9	8	+++	+++	2	3	21	13
	51	10	5	+++	++	1	2	32	23
	52	10	7	++	+++	2	3	40	31
	53	10	6	+++	+++	3	3	35	26
F	54	14	—	+++	0	1	0	31	18
	55	17	6	+++	++	1	0	31	18
	56	17	6	+++	++	2	2	25	9
	57	17	9	+++	+++	1	3	35	19
	58	17	12	+++	+++	3	3	37	21
G	59	26	—	+++	0	2	0	29	4
	60	26	—	+++	0	2	2	30	5
	61	26	—	+++	0	3	0	31	6
H	62	37	5	+++	++	2	1	70	34
	63	37	5	++	+++	2	3	48	12
	64	37	5	+++	++	2	2	48	12
	65	43	8	+++	++	2	2	68	26
	66	43	4	++	++	2	2	48	6
	67	43	7	++	+	2	1	55	13
	68	43	5	++	+++	2	3	49	7

Explanation: P = Primarily ophthalmitic animal; O = Originally healthy partner.

Lesions initiated by our antiserum occurred in all parts of the eye because whole eyes were used for immunization. Their strict limitation to tissues of the eye, however, implies an extraordinary specificity of rabbit tissue antibodies, which is rare in cytotoxins. Rapid development of panophthalmitis after injection suggests the same immediate type of passive and reverse anaphylaxis as has been found in experimental glomerulonephritis of the rat after injection of rabbit nephrotoxins (8,9). Our experiments show furthermore that experimental panophthalmitis was transferred in 21 of 25 pairs from primarily ophthalmic rats to originally healthy partners by parabiosis. Lesions of the eye occurred in the originally healthy animals between 4th and 12th day after parabiosis, irrespective of whether an interval of 8-10 or 37-43 days had elapsed between injection of cytotoxic antiserum into primarily healthy animal and institution of parabiosis. Thus, in the latter group, the first signs of panophthalmitis were recorded in primarily healthy animals between 41st and 50th day after injecting rabbit antiserum into the partner (group H, Table III). Similar observations have been made in our previous experiments in which after intervals of 30-45 days (and recently after 71 days) between injection of rabbit nephrotoxins and parabiotic union, glomerulonephritis has been transferred to originally healthy rats(4,5). The question, therefore, arises whether or not panophthalmitis in originally healthy parabionts was produced by the heterologous rabbit-anti-eye antibodies injected into the primarily ophthalmic animal. Experiments are in progress to elucidate the immunological mechanism of this transfer phenomenon, *i.e.*, whether sensitization of the eye of the originally healthy partner is due to surviving injected heterologous antibody or to some other not yet clearly determined secondary tissue damaging factor.

While the transferred ophthalmitis generally was less pronounced than in the injected animals, the characteristics of inflammatory lesions of the eye were the same. In both, all of parts of eye tissues were affected, and the delayed tuberculin-type of hypersensitivity reaction was indicated by lymphoid and plasma cells in the inflamed areas(10). However, as this type of anaphylaxis has been produced in injected animals by serum antibodies, it cannot be decided whether transfer of ophthalmitis was produced by serum antibodies or antibody containing white cells of the primarily ophthalmitic animal.

Discussions of clinical implications of our findings are beyond the scope of this paper. However, certain similarities are obvious between panophthalmitis produced by injection of anti-eye-antibodies and transferred by means of parabiosis, and the human disease called sympathetic ophthalmia. With respect to whether nervous factors are involved in this disorder, it seems justified to stress that nervous elements cannot be implicated in our experiments. Certainly, this experimental panophthalmitis has been produced only by humoral factors. This recalls the old concept of autoallergic processes in sympathetic ophthalmia(11), production of inflammation of the lens by sensitization with homologous lens material(12) and the newer therapeutic approaches with steroid hormones in this disease(13).

Summary. 1. Experimental panophthalmitis has been produced in 36 of 40 rats injected with antiserum of rabbits against rat eye tissue within 24 hours after injection. Clinical and histological features of eye lesions are described. 2. Twenty-eight rats in which pathological findings were recorded by ophthalmoscopic examination after application of the antiserum ("primarily ophthalmitic animals") were joined in parabiosis with normal animals ("originally healthy partners") 8-43 days after injection. Three rats of the parabionts died after operation. In 21 of the remaining 25 pairs, transfer of panophthalmitis on originally healthy partners occurred between 4th and 12th day after parabiosis and the 11th and 50th day after injection of antiserum into primarily ophthalmitic animals respectively. Ophthalmoscopic and histological findings of transferred ophthalmitis were similar to those induced by injection. 3. The intervals between injection of antiserum into primarily ophthalmitic rat, parabiosis and occurrence of ophthalmitis in the originally healthy partners lead one to ask whether the panophthalmitis in the originally healthy parabionts is due to surviving injected heterologous antibodies or to some other not yet determined secondary tissue damaging factor.

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Serum Non-Esterified Fatty Acids in Patients with Recent Myocardial Infarctions.*† (24665)

JOHN M. PISKORSKI AND EVELYN B. MAN (Introduced by P. K. Bondy)
Dept. of Internal Medicine, Yale University School of Medicine

Triglycerides are frequently elevated in sera of patients with coronary artery disease (1). Since non-esterified fatty acids (NEFA) and triglycerides are closely associated metabolically(2-15) NEFA concentrations were investigated daily in post-absorptive sera from patients who had had myocardial infarctions 10 to 29 days previously.

Methods. Serum NEFA were measured by Dole's method(4,5) with the following alterations: Redistillation of heptane and isopropyl alcohol was discontinued after identical results were obtained with redistilled and undistilled solvents. Solutions of extracted NEFA, stirred with washed nitrogen(5), were titrated with a Rehberg or Gilmont burette(4,5,6) using Nile Blue as the indicator(8). The alkali used for titration was standardized against constant boiling hydrochloric acid. Calculations were made as follows: the observed volume of the upper phase (which exceeds actual volumes of heptane used) was employed as the volume in which titrated NEFA were distributed. The upper phase volumes were checked with every lot of extraction reagents. Such calculations avoid the use of a standard palmitic acid solution which may be inaccurate if crystalline palmitic acid is impure or if temperature alters the volume of the solvent heptane. This method of calculation makes no allowance for the coefficient of distribution of NEFA in the 2 phases of the solvent system. About 150 recovery tests were conducted on heptane solution of palmitic acid alone, or combined with serum or dilutions of Red Cross Albumin to yield concentrations of NEFA throughout the physiological range. NEFA in the upper phase were about 92% of theoretical, even in samples containing more than 1 meq/l. The maximum technical error of the method was less than 0.057 meq/l and is a little higher than data quoted by Dole(4) and Gordon(8) because it includes day-to-day variations. This error is small in comparison with differences in NEFA which have been shown to have physiological significance(2-15). The subjects were 4 non-diabetic individuals (normal post-absorptive blood sugars), otherwise unselected, who had been conducting normal activities until their acute infarcts in July, 1958 just prior to hospitalization (Table I). All were ambulatory during the present studies. After about one month hospitalization all 4 patients returned home in a reasonably healthy state. Changes in weight were not recorded; all patients seemed normally nourished except that C was stocky. After dietary intake had become regular and the effects of heparin treatment on plasma lipids had disappeared, blood was drawn from the antecubital vein each week day morning between 7:15 and 8 a.m. about 12 hours after ingestion of fluids such as fruit juices during the previous evening. Serum NEFA were determined at 24 or 96 hour intervals for 3 to 11 days; serum triglycerides, cholesterol and lipid phosphorus, measured by methods described earlier(1) were estimated in some of the samples for NEFA.

Results. Data on the 4 patients after myocardial infarctions are shown in Table I. All concentrations of NEFA were between 0.203 and 0.592 meq/l which are within the range for normal non-obese non-diabetic young men in a post-absorptive state(5,6). There were only minor variations from day to day in any individual. The greatest difference in NEFA was only 0.328 meq/l for patient A during an interval 15 to 21 days after infarction, when his physical activity was increasing. NEFA of the 4 patients in the hospital were lower

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TABLE I. Serial Serum Lipids of 4 Patients Having Coronary Artery Disease.

Patient	Days after infarction	NEFA	Triglycerides	Total fatty acid	Cholesterol	Lipid phosphorus
		meq/l			mg/100 ml	
A (A6-23-42) ♂, age 50 Posterior wall infarction. Regular diet. No anticoagulants.	11	.207	4.7	12.5	156	8.5
	12	.207	4.9*	13.3	186	
	13	.209				
	15	.203				
	16	.350				
	18	.211	9.2	19.0	190	10.7
	19	.251	8.2	18.2	203	10.6
	20	.441	5.6	15.6	228	9.8
	21	.531	6.2*	16.2	230	
B (49-39-03) ♂, age 66 Antero-lateral infarction. Regular diet. Heparin on admission and next day. Dicoumarol during studies.	10	.366	4.1	13.7	238	8.9
	11	.491	4.6*	13.9	219	
	12	.398	8.8	19.2	211	11.2
	13	.592	6.0	15.2	224	8.6
	14	.428				
	18	.492				
	19	.484				
	20	.356	4.5	13.6	212	8.9
	21	.241	4.7	13.6	228	8.0
C (43-50-02) ♂, age 56 Angina 1950-55. Post. infarction 1955. No angina, no congestive heart failure 1956-7/1958. New post. wall infarction. 1500 cal., 1200 mg Na for 9 days followed by regular diet. Heparin first 3 days. Dicoumarol during studies.	18	.369				
	19	.411	18.2	31.4	273	14.0
	20	.519	15.8	29.6	295	14.2
	21	.591				
	22	.432				
	26	.439				
	27	.504				
	28	.578	22.1	39.0	396	16.3
	29	.539	21.6	38.2	397	15.9
D (49-35-19) ♀, age 53 Ant. infarction 5 days after hospitalization. Digitoxin cont. from 6th day post. infarction. Heparin 5th through 9th day post. infarc. Dicoumarol 5th through 23rd day. 1200 mg Na until regular diet on 17th day post. infarction.	20	.303	6.7	16.1	221	9.2
	21		7.4	18.3	241	11.0
	22	.363				
	23	.379	7.9	18.6	211	11.6
	24	.349				

* Fatty acids of phospholipids calculated from previous lipid phosphorus.

than unpublished values on 8 ambulatory male out-patients, studied at least 3 months after myocardial infarctions. Normal concentrations of NEFA persisted for all 4 patients (Table I) although in each case the triglycerides were higher than the normal maximum of 6.0 meq/l(1) in at least 2 samples of serum, and were persistently elevated in one male, C. In this brief study concentrations of serum NEFA bore no relationship to the previous myocardial infarction or to the elevation of triglycerides which occurs in this disease(1).

The serum lipid fractions on patients 10 to 29 days after a myocardial infarction furnish further examples of data already reported from this department(1) in which it was found that serum cholesterol and fatty acids often rise during recovery from a myocardial infarction. Lipid concentrations determined soon after an infarction may not be representative of the values for that individual 3 to 4 weeks after the acute episode, (*e.g.*, A & C, Table I). The previous observation(1) that serum triglycerides are more frequently elevated than serum cholesterol following a myocardial infarction is confirmed by the fact that triglycerides were above the maximum of the normal range in at least 2 sera obtained at intervals of 24 hours for all 4 patients although cholesterol was above the normal maximum only in the serum of C.

Recent observations suggest the importance of non-esterified fatty acids as fuel for the myocardium. Bragdon and Gordon(3) found a high content of labelled unesterified fatty acids in the heart, after injection of this material into carbohydrate fed rats. Korn has reported that myocardium has a high content of the enzyme lipase(11), but presence of this enzyme in normal plasma has not been proven. If injury such as an infarction results in release of myocardial transaminase into the blood, lipase may also be liberated after an acute infarction. At the time when transaminase concentrations are increased, changes in NEFA concentrations due to lipase would be under the influence both of heparin (if this were prescribed) and of the decreased caloric intake which occurs shortly after an infarction. To avoid these confusing effects on NEFA the present patients were observed only after transaminase would have fallen, and some days after cessation of medication with heparin and resumption of regular intake of food.

Few data have been published concerning NEFA concentrations of one individual studied repeatedly in a post-absorptive state. Both Gordon(8) and Dole(4) observed increases in plasma NEFA during the forenoon when duration of an overnight fast was prolonged. In view of the extremely rapid turnover rate of NEFA(3,11) (2 minutes half life in the dog(2)) it is difficult to appraise the significance of isolated NEFA values. The variation in concentrations of NEFA in arterial and venous plasma found by Gordon (8), may have resulted from changes in supply and demand for NEFA. Our data indicate independence of plasma NEFA and of plasma triglyceride concentrations. Utilization of exogenous triglycerides without obligatory retransport as unesterified fatty acid has been found(16). Constancy of NEFA of our 4 patients, on restricted muscular activity and receiving caloric intake from hospital meals at regular intervals, clearly indicates the importance of evaluating NEFA concentrations in relation to the time since the last meal and the amount of exercise during that interval.

Summary. By a slight modification of Dole's method serum NEFA of 4 patients were measured at 24 hour intervals 10 to 29 days after myocardial infarction. These subjects were in a post-absorptive state. Values varied within a narrow, normal range from 0.203 to 0.592 meq/l. Serum NEFA concentrations were not correlated with serum triglycerides; the latter increased above the maximum for normal males.

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Oxidative Metabolic Pattern of Normal and Ketotic Cow Liver Incubated With 1-C^{14}-Labeled Aliphatic Acids.* (24666)

A. C. CHUNG, F. G. HUETER AND J. C. SHAW
Dept. of Dairy Husbandry, University of Maryland, College Park

It is well accepted that the ruminant derives the major portion of its energy from volatile fatty acids (VFA) produced in the rumen as a result of fermentation of ingested feed by rumen microflora. By and large, the VFA produced in the rumen are absorbed as such and reach the liver directly through the portal system. The major VFA concerned are acetic, propionic, butyric and valeric acids, listed in their normal descending order of production and absorption. In addition, formic acid is also an important metabolite for ruminants since we showed it was produced in rather marked amounts by perfused goat livers(4). If one assumes that the ruminant liver is very similar in its behavior, in a given set of conditions, to the liver of the monogastric animal, then the major metabolic dissimilarities between these animals must arise from differences in substrates available to livers through the portal blood (glucose for monagastrics and VFA for ruminants) and in any enzymatic and endocrine adaptation to these differences. In addition it is commonly accepted that liver function is disturbed(5,6) when ruminants, particularly dairy cattle, exhibit clinical ketosis, a primary metabolic disorder or syndrome believed associated with stress(7). For above reasons, studies were initiated concerning oxidative metabolic pathways of various VFA by normal and ketotic ruminant liver slices.

Methods. The methods used including chromatographic - autoradiographic technic were described by Katz and Chaikoff(3) and Brown *et al.*(1). Independent incubations of liver slices (0.5 g) were made in duplicate with the following 1-C^{14} labeled acids: formic, acetic, propionic, butyric and valeric. For each incubation, 25 μM of substrate containing 25 μc of C^{14} was employed. The flasks were incubated 2 hours at 39 and 37°C for cow and rat liver respectively. The relative position of non-volatile compounds separated by 2-dimensional chromatography is shown in Fig. 1.

Results. In all incubations the volatile fraction contained the largest percentage (83-98%) of recovered C^{14}. Since this fraction represents non-utilized substrate, it can be assumed that large excesses of substrate were present during all incubations. In addition, no significant lipogenesis was observed during incubations.

Formate-C^{14}. Formate carbon was incorporated to greatest extent into glucose, followed by what appear to be phosphate esters (Table I). A marked decrease occurred in $C^{14}O_2$ production and in incorporation of formate into non-volatile compounds in ketotic cow liver slices. Compared to the nor-

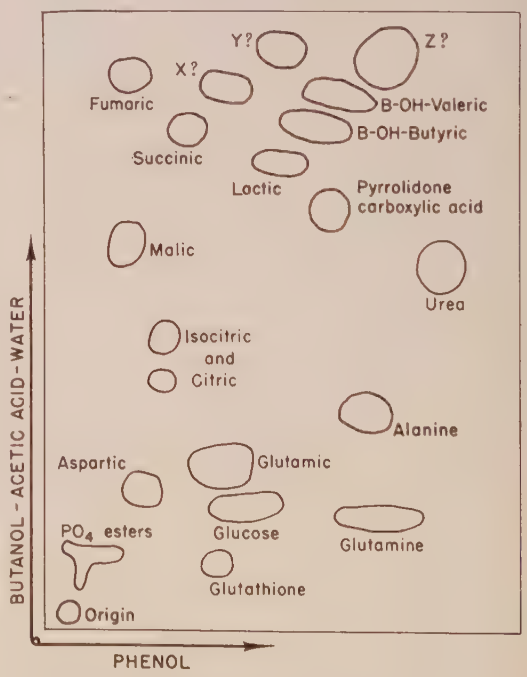

FIG. 1. Diagrammatic representation of location and identity of radioactive spots observed on various autoradiograms.

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TABLE I. Percentage Incorporation of Isotope from Formate-C^{14} into Chromatographic Nonvolatile Compounds and Various Major Fractions by Liver Slices.

	Liver sources:							
	"Normal" cow*		Ketotic cow A		Ketotic cow B		Rat	
	Flask		Flask		Flask		Flask	
	I	II	I	II	I	II	I	II
Glucose	55.3	55.2	82.4	83.3	31.8	35.2	59.6	62.9
β-Hydroxybutyrate	3.7	2.2	.0	.0	5.8	7.0	Tr	.0
β-Hydroxyvalerate	.0	.0	.0	.0	8.3	4.9	.0	.0
Malate	4.6	2.7	.0	.0	Tr	Tr	3.8	3.2
Aspartate	.0	.0	.0	.0	21.7	11.1	.0	4.9
Glutamate + glutamine	7.2	7.1	.0	.0	9.4	7.7	3.0	2.9
Isocitrate + citrate	1.3	2.5	.0	.0	Tr	Tr	.0	.0
Lactate	3.5	3.8	.0	.0	4.5	4.9	4.2	3.6
Alanine	.0	.0	.0	.0	.0	.0	2.9	.0
Succinate	2.2	1.8	.0	.0	.0	.0	Tr	.0
Fumarate	Tr	Tr	.0	.0	.0	.0	.0	.0
PO_4 esters	8.1	10.4	Tr	Tr	6.4	16.1	13.0	11.3
Urea	4.9	3.6	.0	.0	Tr	5.9	5.5	7.0
Glutathione	.0	.0	.0	.0	.0	.0	3.4	.0
Unidentified	2.3	3.1	.0	.0	.0	.0	.0	.0
Origin	5.5	6.9	Tr	Tr	Tr	Tr	Tr	4.2
Total chromatogram radioactivity (cpm)	2,308	1,699	158	140	668	603	1,405	1,263
Major fractions	% of recovered C^{14}							
Respired CO_2	1.9	2.0	.5	.4	2.2	1.4	1.0	1.2
Nonvolatile fraction	2.4	3.5	3.3	1.3	2.4	5.2	1.1	1.2
Volatile "	95.7	94.5	96.2	98.3	95.4	93.4	97.9	97.6
Total recovered C^{14} (cpm × 10^6)	14.0	14.0	4.0	3.8	3.5	3.6	15.0	16.0

* Liver sample obtained by biopsy. Tr = Trace.

TABLE II. Percentage Incorporation of Isotope from Acetate-1-C^{14} into Chromatographic Nonvolatile Compounds and Various Major Fractions by Liver Slices.

	Liver sources:							
	"Normal" cow-S*		Ketotic cow A		Ketotic cow B		Rat	
Chromatographic compounds	Flask		Flask		Flask		Flask	
	I	II	I	II	I	II	I	II
Glucose	4.8	4.2	18.7	16.9	11.0	11.3	6.0	5.0
β-hydroxybutyrate	48.0	48.5	12.5	6.1	31.6	32.9	50.1	53.1
β-hydroxyvalerate	.0	.0	5.6	12.4	.0	Tr	.0	.0
Malate	7.5	6.3	7.7	7.5	6.8	7.0	5.3	6.2
Aspartate	7.5	9.5	9.7	7.4	11.2	12.8	3.5	3.7
Glutamate + glutamine	15.2	16.9	12.0	21.8	11.8	14.0	13.9	15.6
Isocitrate + citrate	10.5	11.0	19.7	16.9	13.8	9.9	8.3	6.2
Lactate	4.2	Tr	Tr	Tr	2.5	Tr	Tr	Tr
Alanine	.0	.0	.0	.0	.0	.0	"	"
Succinate	.0	Tr	Tr	Tr	3.6	Tr	2.0	1.6
Fumarate	.0	.0	"	"	Tr	"	Tr	Tr
PO_4 esters	.0	.0	5.0	"	.0	"	"	"
Urea	.0	Tr	.0	.0	.0	.0	2.9	2.7
Glutathione	.0	.0	.0	.0	.0	.0	Tr	Tr
Pyrrolidone carboxylic acid	.0	.0	Tr	Tr	2.5	2.9	.0	.0
Origin	Tr	.0	"	"	3.1	2.1	Tr	Tr
Total chromatogram radioactivity (cpm)	720	874	875	1,106	1,452	1,594	2,198	2,780
Major fractions	% of recovered C^{14}							
Respired CO_2	.4	.3	.1	.1	.1	.1	.1	.2
Nonvolatile fraction	1.7	2.1	1.4	1.5	2.1	2.7	2.5	3.2
Volatile "	97.9	97.6	98.5	98.4	97.8	97.2	97.4	96.6
Total recovered C^{14} (cpm × 10^6)	4.8	4.9	5.8	5.7	5.4	5.1	5.9	6.1

* Liver sample obtained from abbatoir.

TABLE III. Percentage Incorporation of Isotope from Butyrate-1-C^{14} into Chromatographic Nonvolatile Compounds and Various Major Fractions by Liver Slices.

	Liver sources:								
	“Normal” cow-S*		“Normal” cow†		Ketotic cow A		Ketotic cow B		Rat
Chromatographic compounds	Flask I	Flask II	Flask I	Flask II	Flask I	Flask II	Flask I	Flask II	I
Glucose	10.1	9.8	.0	Tr	.0	Tr	Tr	Tr	.0
β-hydroxybutyrate	74.3	74.8	77.0	76.5	57.1	36.9	47.0	48.6	77.4
β-hydroxyvalerate	.0	.0	1.9	2.6	5.2	9.4	4.3	4.7	1.6
Malate	2.8	2.8	2.2	1.6	2.2	2.3	3.4	4.0	2.0
Aspartate	3.3	4.7	2.9	3.3	8.5	11.7	13.0	12.4	1.0
Glutamate + glutamine	4.9	5.5	11.7	12.4	21.9	29.7	21.7	21.7	10.1
Isocitrate + citrate	.0	.0	Tr	1.1	Tr	Tr	Tr	Tr	Tr
Lactate	Tr	1.4	”	Tr	”	”	3.3	3.5	”
Alanine	”	.0	.0	.0	.0	.0	Tr	.0	”
Succinate	”	Tr	Tr	Tr	Tr	Tr	”	Tr	”
Fumarate	.0	.0	”	”	”	.0	”	”	”
X ?	.0	.0	”	”	”	Tr	1.3	”	”
Y ?	.0	.0	1.2	1.1	.0	”	.0	.0	.0
Urea	.0	.0	.0	.0	.0	”	Tr	Tr	2.2
Glutathione	.0	.0	.0	.0	.0	.0	.0	.0	Tr
Pyrrolidone carboxylic acid	.0	.0	.0	.0	.0	Tr	Tr	Tr	”
Origin	Tr	.0	Tr	Tr	Tr	”	”	”	”
Total chromatogram radioactivity (cpm)	2,049	3,324	6,193	5,905	1,717	1,281	3,347	2,604	4,639
Major fractions				% of recovered C^{14}					
Respired CO_2	.4	.6	.5	.5	.3	.4	.7	.6	.4
Nonvolatile fraction	3.9	4.9	13.6	13.4	3.9	3.6	6.3	6.0	8.8
Volatile ”	95.7	94.5	85.9	86.0	95.8	96.3	93.0	93.4	90.7
Total recovered C^{14} (cpm × 10^6)	3.7	3.8	3.1	3.0	3.3	3.2	3.1	3.3	3.5

* Liver sample obtained from abbatoir. † Liver sample obtained by biopsy.

mal cow, the ketotic cow B liver slives incorporated a smaller % of formate into glucose and a larger % into aspartate, glutamine, β-hydroxybutyric acid (BHBA) and β-hydroxyvaleric acid (BHVA). Of the 2 ketotic cows, cow B exhibited the most marked hypoglycemia and ketonuria. The different distribution pattern observed in the case of ketotic cow A may have been due to extremely low incorporation of C^{14} (159 cpm) into the non-volatile components.

Acetate-C^{14}. Total incorporation of acetate label into the non-volatile components was low (Table II). This agrees with results by McCarthy *et al.*(4) which showed that labeled acetate passes through the perfused goat liver with only slight utilization. The data are similar to those observed for rat liver by Katz and Chaikoff(3) and mouse liver by Brown *et al.*(1), except for a lesser amount of label in glucose. The principal difference between normal and ketotic cows was in the higher incorporation into glucose and the lower incorporation into BHBA of labeled acetate by ketotic cow liver slices. The increased incorporation of acetate label into glucose by the ketotic liver slices is of interest in connection with the observation that hypoglycemia in ketotic cows can be ameliorated by oral administration of acetate (7).

Butyrate-1-C^{14}. Results from rat liver slice incubations with butyrate are in close agreement with those of normal cow liver slices (Table III). No marked differences in labeled CO_2 production were noted for the various liver slices. On the average, normal cow liver incorporated about twice as much butyrate into non-volatile compounds as ketotic cow liver. BHBA and the glutamate-glutamine group accounted for the bulk of the label from butyrate with lesser amounts occurring in aspartate, malate and BHVA. Very slight incorporation into C_3 intermediates was observed. The major differences between normal and ketotic cow liver were in incorporation of isotope into non-volatile compounds. Expressed as percentage incorporation, ketotic liver, compared to normal, produced (a) one third less BHBA, (b) twice the relative amount of glutamate-glutamine and BHVA, and (c) 3 times the relative amount of aspartate. Only in the case of the abbatoir cow were significant amounts of label from butyrate found in glucose. This may have been a physiological response to fasting since this animal had been fasted more than 24 hours. Comparing the results obtained with butyrate with those of acetate would suggest that degradation of butyrate into 2 acetate units is far from the complete story concerning metabolism of butyrate by bovine liver slices.

Propionate. Malate, aspartate and lactate contained most of the label from propionate with lesser amounts in BHVA, BHBA and succinate (Table IV). The unidentified compounds designated as X? and Y? (Fig. 1) also contained an appreciable portion of the label. Lower incorporation of label into malate was obtained with ketotic than with normal liver slices. A high incorporation of label was observed in the succinate and X and Y compounds of ketotic cow A.

Valerate-1-C^{14}. The most striking observation with this substrate was its wide distribution into non-volatile intermediates and its marked utilization by cow liver slices (Table V). BHVA and BHBA contained most of the label from valerate except for the ketotic cows in which the label in BHBA was low. It is of considerable interest to note (Table II) that incorporation of acetate label into BHBA by ketotic cow liver tissue was also low. The incorporation of valerate label into malate and glutamate (plus glutamine) was also lower for the ketotic cows. Perhaps the major difference between normal and ketotic liver with this substrate was its large incorporation into an unidentified compound, designated Z (Fig. 1), with negligible incorporation into this compound by normal cow liver slices. The spotting of this substance on the radiogram encompassed the greatest total area, suggesting a large turnover. A slight incorporation of labeled propionate into this substance by ketotic liver slices will be noted in Table IV. Identification of this compound may provide an important clue to the etiology of bovine ketosis. As previously stated, ketotic liver slice incubations resulted in exceptionally marked decreases in oxidation and incorporation of formate. This altered formate metabolism must be an important part of any metabolic explanation concerning boving ketosis.

TABLE IV. Percentage Incorporation of Isotope from Propionate-1-C^{14} into Chromatographic Nonvolatile Compounds and Various Major Fractions by Liver Slices.

	Liver sources:				
	"Normal" cow-S*	Ketotic cow A		Ketotic cow B	
Chromatographic compounds	Flask I	Flask I	II	Flask I	II
Glucose	1.0	.0	Tr	Tr	Tr
β-hydroxybutyrate	Tr	3.4	3.1	2.6	3.3
β-hydroxyvalerate	1.6	4.3	2.4	5.8	2.1
Malate	34.0	9.7	6.4	16.8	17.5
Aspartate	25.9	24.0	18.8	35.6	35.3
Glutamate + glutamine	2.0	1.6	1.6	2.1	2.0
Isocitrate + citrate	.0	Tr	1.3	4.8	2.5
Lactate	6.1	9.4	5.1	8.7	8.1
Alanine	1.1	Tr	Tr	3.1	1.9
Succinate	3.0	9.2	11.2	3.6	4.3
Fumarate	5.1	2.0	Tr	2.8	2.7
X ?	7.6	17.6	23.3	5.5	6.1
Y ?	10.4	16.4	21.5	6.1	6.5
Z ?	.0	.0	Tr	.0	5.0
PO_4 esters	.0	.0	.0	Tr	Tr
Urea	Tr	Tr	Tr	1.3	1.3
Glutathione	"	.0	"	.0	Tr
Origin	"	Tr	"	Tr	"
Total chromatogram radioactivity (cpm)	3,317	2,937	2,241	8,454	7,308
Major fractions		% of recovered C^{14}			
Respired CO_2	.3	.1	.1	.2	.2
Nonvolatile fraction	3.3	1.7	1.4	4.0	3.2
Volatile fraction	96.4	98.2	98.5	95.8	96.6
Total recovered C^{14} (cpm × 10^6)	4.6	13.0	13.0	13.0	13.0

* Liver sample obtained from abbatoir.

It should be noted that the ketotic liver slices resulted in increased incorporation of formate, acetate and butyrate into aspartate. An increased production of glutamate was also observed for ketotic liver slices incubated with labeled butyrate. This increased production of aspartate and glutamate may be related to faulty gluconeogenesis in ketotic cow liver slices. This is of interest in relation to the reports of Shaw(7) concerning the

TABLE V. Percentage Incorporation of Isotope from Valerate-1-C^{14} into Chromatographic Nonvolatile Compounds and Various Major Fractions by Liver Slices.

	Liver sources:					
	"Normal" cow*		Ketotic cow A		Ketotic cow B	
	Flask		Flask		Flask	
Chromatographic compounds	I	II	I	II	I	II
Glucose	Tr	Tr	Tr	Tr	.5	.7
β-hydroxybutyrate	27.5	23.9	4.1	2.7	5.5	7.0
β-hydroxyvalerate	43.8	46.9	57.8	52.8	48.7	42.7
Malate	3.6	3.1	.8	1.1	1.4	1.6
Aspartate	4.3	3.9	2.1	4.4	4.0	4.7
Glutamate + glutamine	9.8	10.0	4.0	5.9	6.0	5.5
Isocitrate + citrate	1.8	2.7	2.3	3.4	2.6	2.2
Lactate	1.1	1.1	.8	Tr	1.9	1.7
Alanine	.0	Tr	Tr	.0	Tr	Tr
Succinate	1.0	1.0	1.6	5.0	1.2	"
Fumarate	.7	.8	.0	.0	Tr	"
X ?	1.1	1.1	3.7	10.2	2.7	1.2
Y ?	3.0	2.7	.0	3.4	2.5	3.2
Z ?	Tr	Tr	18.7	6.7	19.5	25.7
PO_4 esters	"	.6	Tr	Tr	Tr	Tr
Urea	.7	.5	2.1	1.8	1.3	1.1
Pyrrolidone carboxylic acid	.6	.7	1.1	1.4	1.2	.9
Origin	Tr	.5	Tr	Tr	Tr	Tr
Total chromatogram radioactivity (cpm)	12,179	10,718	9,195	5,474	9,545	10,178
Major fractions			% of recovered C^{14}			
Respired CO_2	.5	.5	.4	.4	.4	.4
Nonvolatile fraction	5.6	5.0	3.7	3.4	3.8	4.0
Volatile "	93.9	94.5	95.9	96.2	95.8	95.6
Total recovered C^{14} (cpm × 10^6)	13.0	14.0	13.0	13.0	13.0	13.0

* Liver sample obtained by biopsy.

effective treatment of ketosis by administration of ACTH and glucocorticoids.

The overall decreased metabolic activity *in vivo* studies by Robertson *et al.*(6) and Peeters *et al.*(5).

Conclusions. 1) The oxidative metabolic patterns of normal and ketotic cow liver studies with 1-C^{14}-labeled C_1 through C_5 aliphatic acids, were determined using a chromatographic-autoradiographic technic. 2) The label from each metabolite had its own distinctive distribution pattern as evidenced by autoradiograms. 3) Normal cow liver slices incorporated carboxyl-labeled carbons of the various aliphatic acids, to the greatest extent, into non-volatile compounds as follows: formate, into glucose and what appear to be phosphate esters; acetate, into β-hydroxybutyrate, glutamate, citrate and small amounts of glucose and lactate; butyrate, into β-hydroxybutyrate and glutamate-glutamine with little incorporation into C_3 intermediates; propionate, into malate, aspartate and lactate; valerate, into β-hydroxybutyrate and β-hydroxyvalerate with a very wide distribution into the other intermediates chromatographically obtainable. 4) Ketotic cow liver slices exhibited a marked reduction in utilization of formate and an altered distribution in labeling of intermediates when incubated with various labeled aliphatic acids.

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Effect of Whole Body X-Irradiation on Plasma Iron Turnover in Rats.* (24667)

Eb C. Girvin†‡ and John K. Hampton, Jr.
Dept. of Physiology, Tulane University School of Medicine, New Orleans, La.

That irradiation depresses mitotic activities is well known. Bone marrow cells are particularly sensitive and many investigations, as reviewed by Jacobson(1), have produced evidence of depression of erythropoiesis following irradiation. Most studies have been made by examination of the erythroid elements of the bone marrow, disappearance of reticulocytes from the circulating blood, or reduction in uptake of injected radioiron by newly formed red cells(1,2). Since Bothwell and co-workers(3) have shown that plasma radioiron turnover is depressed following radiation, and others(4-8) have shown the validity of plasma iron turnover rate as an index of erythropoiesis, we have used this method to quantify the sensitivity of the bone marrow to irradiation and to study the depression and recovery of its activity.

Materials and methods. We have used male CFW rats weighing 325-475 g and Holtzman rats weighing between 250 and 375 g. They were given Wayne lab-blox and tap water *ad libitum.* In preliminary experiments food was withheld 24 hours before determining plasma iron turnover; however, it was found that failure to do so did not affect the results. Thus, the remainder of the experiments were done without withholding food. *Radioiron disappearance* measurements were made by injection of .1 ml of iron citrate solution containing a maximum of 1.5 μg of chemical iron (1-2 microcuries Fe^{59}) into the femoral vein of a nembutalized rat. Four serial blood samples of .3 ml were taken from the opposite femoral vein at 15- to 30-minute intervals after injection. These samples were centrifuged and 100 lambda of plasma was pipetted for determination of radioactivity. Radioactivity measurements were made in a well-type scintillation counter using a sodium iodide thalium-activated crystal. The radioactivity was plotted on semi-logarithmic paper against time and the T ½ value was computed from the disappearance curve. *Radiation* was administered with a Westinghouse duo-condex X-ray machine at 200 KVP, 12.5 ma, ½ mm Cu, and 1 mm A1 added filtration. Calibration was done with a 250 r thimble of the Victoreen instrument at 40 cm TSD in air with a dose rate of 58 to 62 r per minute. The animals were restrained during irradiation in individual perforated lucite boxes on a turntable run at 3 rpm. *Plasma iron* determinations were made by the method of Schade *et al.*(9) on samples of plasma drawn after the last sample was taken for determination of radioiron disappearance. All radioiron disappearance and plasma iron measurements were run between 1:00 p.m. and 3:00 p.m., to avoid effects of diurnal variations(8).

Results. I. CFW rats were exposed to irradiation doses of 50, 150, and 300 r, and T ½ values for Fe^{59} disappearance were determined 1, 7, 13, and 21 days following irradiation. The results are shown in Fig. 1. Each observation represents an average of 4-6 animals. Control T ½ values were determined on 6 rats and ranged from 69-93 min. The extended T ½ values indicate that erythropoiesis is depressed at 24 hours after all doses of irradiation. Amount of depression is directly related to dose. At 7 days T ½ values have returned to the control level. This rapid and apparently complete recovery continues through 14 and 21 days.

II. A preliminary experiment using CFW rats was carried out following 450 r. T ½'s were determined at 6, 12, 18, 48, 66, and 96 hours post-irradiation. The results showed that bone marrow depression, as reflected in T ½, was rapid and sufficiently uniform for the period of maximum depression to be de-

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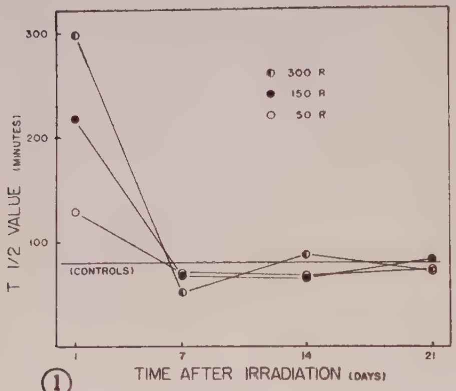
‡ Permanent address: Dept. of Biology, Southwestern Univ., Georgetown, Texas.



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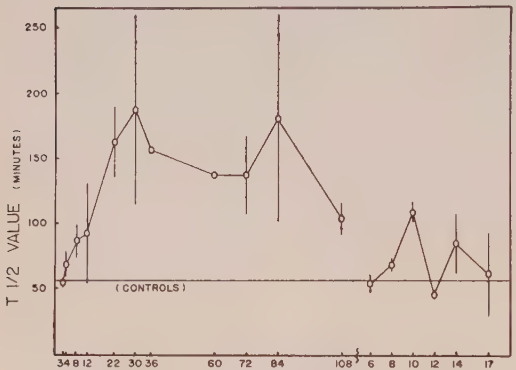

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FIG. 1. T ½ value for disappearance of Fe^{59} from plasma of CFW rats at different times following various doses of X-ray. 4-6 rats/datum.

FIG. 2. T ½ value (± S.D.) for disappearance of Fe^{59} from plasma of Holtzman rats at various times following 450 r X-irradiation. 2-5 rats/datum except at 36 and 60 hr, which points represent single animals.

termined. Thus, in a later experiment Holtzman rats, instead of CFW, were exposed to 450 r and examined at frequent intervals from 3 hours to 17 days post-irradiation. Evidence of bone marrow depression appeared within 4-8 hours after irradiation and proceeded rapidly to 24 hours (Fig. 2). Maximal depression occurred between 24 and 48 hours and the largest deviation around mean values was observed during this period. Rapid recovery proceeded from the 3rd and 4th day to the 5th or 6th day. Variations in T ½ were present from the 6th to 17th days.

Discussion. These experiments have confirmed the findings of many others that bone marrow activity is depressed following irradiation(1). We have used a method (disappearance of radioactive iron from the plasma) that is especially sensitive and suitable for carrying out time course observations since disappearance rate reflects bone marrow activity during the one-hour period of sampling. We have shown that a marked diminution in turnover rate of plasma iron is seen 24 hours after irradiation. Furthermore, depression of iron turnover rate is evident by 6 hours post-irradiation. While it has long been known that leukocytes show an early post-irradiation reduction(1), it has been less obvious that bone marrow erythrogenic cells responded so quickly. Due to the long life span of red cells, lack of production of cells for many days is required to cause a conspicuous reduction in red cell count.

Of equal interest is the fact that red cell production rate, as indicated by plasma iron turnover, appears to have returned to normal by the sixth day. A period of anemia is commonly observed, which is prominent during the second post-irradiation week. That hemorrhagic diathesis and plasma volume changes have contributed to this anemia has been shown by Kahn and Furth(10). Our data distinctly show early depression and early functional recovery of bone marrow and thus confirm the conclusion that the anemia may be largely relative to factors other than erythropoiesis.

In other laboratories the importance of determining plasma iron level has been emphasized(3,4,8). We agree with the necessity for realizing that plasma iron turnover is a function both of turnover rate and the size of plasma iron mass. Accordingly, we determined plasma iron chemically and calculated plasma volumes of animals by extrapolation of the disappearance curves(7). Although Chanutin and Ludwig(11) reported hyperferremia in rats following 500 r, the plasma iron mass of the animals in our experiments varied so little that T ½ values remained the substantial reflection of iron usage.

We believe that measurement of the T ½ value in the period 24 to 48 hours post-irradiation is a sensitive and reasonably quantitative indication of bone marrow depression following irradiation. Our experiments suggest that this approach would produce data more reliable than red cell uptake of radioiron, especially during weak or short-lived effects. Hodgson *et al.*(12) have reached the same conclusion following use of this method to study bone marrow stimulation. These experiments are being extended to determine the limits of accuracy and sensitivity of this method.

Summary. We studied the depression of plasma radioiron turnover rates in rats following X-irradiation. The depression is prompt (maximal by 24-30 hours) and, at 24 hours post-irradiation, is directly related to dose between 50 r and 300 r. Recovery occurs by the 6th post irradiation day, indicating that subsequent anemia is more likely due to hemorrhagic diathesis than bone marrow dysfunction. Sensitivity of the radioiron turnover method permitted a study of bone marrow response at frequent short intervals after irradiation.

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Potentiation of Toxicity of Malathion by Triorthotolyl Phosphate.* (24668)

SHELDON D. MURPHY, ROBERT L. ANDERSON AND KENNETH P. DUBOIS
Dept. of Pharmacology, University of Chicago

The demonstration by Frawley and associates(1,2) that potentiation of toxicity results from simultaneous administration of ethyl p - nitrophenyl thionobenzenephosphonate (EPN) and S-(1,2-dicarbethoxyethyl) - O,O-dimethyl phosphorodithioate (malathion) to rats and dogs stimulated other investigations (3,4) to ascertain whether various pairs of insecticidal organic phosphates cause a similar effect. In view of widespread use of these insecticides on food crops, the testing of each organic phosphate in combination with each of the others currently used, is now an established procedure essential from standpoint of protection of public health. Potentiation of toxicity which occurs with EPN and malathion has recently been explained by Cook *et al.*(5) and Murphy and DuBois(6) on the basis of ability of EPN to inhibit detoxification of malathion. Detoxification of malathion consists of hydrolysis of 1,2-dicarbethoxyethyl side chains(5,7) and this reaction is catalyzed by non-specific esterase present in liver and some other tissues(6). Under these circumstances it seemed likely that any chemical agent capable of inhibiting non-specific tissue esterases could potentiate toxicity of malathion. Our attention was attracted to finding of Myers and Mendel(8) that triorthotolyl phosphate (TOTP) inhibits aliesterase activity of livers of rats. This compound has the chemical structure shown on page 484. The present investigation was conducted to ascertain whether this agent, used industrially

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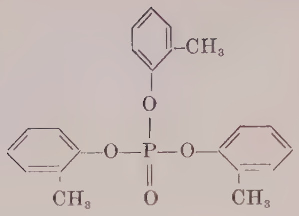

as plasticizer, potentiates toxicity of malathion through inhibition of its detoxification.

Methods and materials. Adult male Sprague-Dawley rats (250-350 g) were maintained in air-conditioned laboratories at 65-75° F and fed Rockland Rat Diet. TOTP was dissolved in sesame oil for intramuscular administration and given intraperitoneally in undiluted form at higher dosage levels or as propylene glycol solution when low doses were administered. For cutaneous application of TOTP the undiluted compound was spread evenly over a circumscribed area 4.5 cm diameter on shaved backs of rats. When doses of less than 250 mg/kg of malathion were given, the compound was dissolved in 10% ethanol and 90% propylene glycol and concentration of malathion was adjusted so that an amount equivalent to less than 0.3% body weight was injected. Doses exceeding 250 mg/kg of malathion were given in undiluted form. In toxicity studies, the animals were observed for 10 days after treatment and LD_{50} values were calculated from mortality data by logarithm-probability method. Cholinesterase measurements were performed manometrically by the method of DuBois and Mangun(9) and measurements of enzymatic detoxification of malathion were made, using the method of Murphy and DuBois(6).

Results. Prior to potentiation tests the acute toxicity of TOTP and malathion to male rats was measured. LD_{50} values were 2500 mg/kg for TOTP and 1100 mg/kg for malathion when the compounds were administered in single doses intraperitoneally. Doses of malathion near the LD_{50} produced typical symptoms of poisoning by cholinergic organic phosphates which began in 15 to 30 minutes after injection and resulted in death following lethal doses in 1 to 3 hours. Symptoms produced by TOTP consisted of general debility, salivation and severe diarrhea but mortality was not observed for at least 18 hours. Differences in time of occurrence of symptoms and death aided in subsequent experiments in determining which compound was the potentiating agent.

Myers and Mendel(8) found that intramuscular injection of 0.1 ml of TOTP on alternate days for 5 days caused 97% inhibition of aliesterase activity of liver and serum of rats. Our initial experiments on the influence of TOTP on toxicity of malathion consisted of administration of 3 intramuscular doses of 440 mg/kg of TOTP on alternate days followed by one-half of the LD_{50} (550 mg/kg) of malathion given intraperitoneally 24 hours after last dose of TOTP. All 3 animals given this treatment died within one hour after malathion, whereas no mortality occurred in controls which received either drug alone. When 3 intramuscular doses of 110 mg/kg were administered, intraperitoneal administration of 550 mg/kg of malathion caused 100% mortality when given 24 hours after last dose of TOTP. When dose of TOTP was reduced to 10 mg/kg, death of all 5 rats given 550 mg/kg of malathion occurred. The particular solvent used for TOTP and malathion was not an important factor in producing potentiation since TOTP (110 mg/kg) given in undiluted form or as sesame oil or propylene glycol solutions caused 100% mortality of rats given 550 mg/kg of malathion 24 hours later. Then a more extensive study was made of the effect of TOTP on susceptibility of rats to acute toxic effects of malathion. Various routes, doses and dosage schedules were used for TOTP. The results are summarized in Table I. TOTP markedly increases susceptibility of rats to malathion. Potentiation occurred regardless of route of administration of TOTP which is explainable on the basis of subsequent experiments which demonstrated that TOTP inhibits detoxification of malathion. The greatest increase in susceptibility was noted when malathion was given 24 hours after intraperitoneal or cutaneous administration of TOTP. The greater toxicity of malathion to rats given TOTP 24 hours earlier as compared with simultaneous administration of the 2 agents indicated that relative time of administration of the 2 agents is

TABLE I. Effect of TOTP on Susceptibility of Rats to Malathion.

TOTP dosage, route of admin.	Time of admin. of malathion	No. of animals	Approx. LD_{50}* of malathion (mg/kg)
No TOTP		32	1100
10 mg/kg, intramusc. on alternate days for 5 days	24 hr after last inj. of TOTP	25	125
110 mg/kg, cutan., single application	24 hr after TOTP	34	12.5
110 mg/kg, intraper.	*Idem*	28	8.2
Idem	Simultaneously with TOTP	22	61
10 mg/kg, intraper.	*Idem*	25	175

* Malathion administered intraper. in all cases.

an important factor in determining degree of potentiation. Deaths usually occurred within 30 to 60 minutes after administration of malathion and TOTP, which corresponds with time of death of animals treated with large doses of malathion alone. No animals treated with TOTP exhibited any untoward effects until malathion was administered. These observations indicated that the only significant action of TOTP was to increase susceptibility of rats to malathion. Further evidence in support of this idea was obtained by experiments in which sequence of administration of malathion and TOTP was varied. When 110 mg/kg of TOTP were given intraperitoneally to 5 male rats 24 hours before injection of 550 mg/kg of malathion, symptoms of malathion poisoning developed rapidly and death of all animals occurred. In contrast, when malathion was administered 24 hours before TOTP, no symptoms or mortality were observed. Evidence that the potentiating action of TOTP is of long duration was furnished by experiments in which 550 mg/kg of malathion were given to groups each containing 5 rats at 1, 3, 7 and 14 days after single intraperitoneal dose of 110 mg/kg of TOTP. One hundred % mortality occurred within 1 hour after administration of malathion in all groups.

Large doses of TOTP are known(10) to inhibit cholinesterase. It was, therefore, considered advisable to initiate our study of the mechanism of increased toxicity resulting from simultaneous administration of malathion and TOTP, by determining whether or not additive inhibitory effects by the 2 compounds occurred. Cholinesterase measurements on brain, submaxillary glands and serum of 3 rats showed that no inhibition of enzyme activity occurs at 24 hours after 3 intramuscular injections of 110 mg/kg of TOTP or after a single cutaneous application of 110 mg/kg of TOTP. In another experiment, groups each containing 3 male rats were given intraperitoneal injections of 110 mg/kg of TOTP, 150 mg/kg of malathion or both agents simultaneously. The animals were sacrificed 30 minutes after treatment and cholinesterase assays were performed on brain, submaxillary glands and serum. Average cholinesterase activity of tissues from 4 untreated rats which served as controls was 100.5 for brain, 24.9 for submaxillary glands and 7.4 for serum expressed in terms of microliters of CO_2/50 mg of tissue/10 minutes. The results of these measurements are summarized in Table II. Cholinesterase activity was not appreciably affected by doses of malathion or TOTP employed when the compounds were given separately. However, simultaneous administration of both compounds at the same dosage levels resulted in marked inhibition of cholinesterase activity of all tissues.

The existing evidence(5,6) that EPN potentiates toxicity of malathion by inhibiting its detoxification suggested that a similar mechanism was responsible for potentiation of toxicity observed with TOTP and malathion, particularly since TOTP is a strong inhibitor of non-specific liver esterases(8). This possibility was tested by measuring the effect of TOTP on enzymatic detoxification of malathion using the method of Murphy and

TABLE II. Effect of Malathion and TOTP Separately and in Combination on Cholinesterase Activity of Rat Tissues.

Compound	Dose (mg/kg)	% of control activity Brain	 Submaxillary gland	 Serum
TOTP	110	100.5	91.2	81.1
Malathion	150	100.3	97.2	67.6
TOTP* Malathion	110 150	40.3	49.8	40.1

* TOTP and malathion given simultaneously.

DuBois(6). For this experiment 3 male rats were given 110 mg/kg of TOTP intraperitoneally. The animals were sacrificed 30 minutes later and liver and serum were assayed for ability to detoxify the oxygen analogue of malathion. Assays were conducted on tissues of 3 untreated controls for comparison. Liver and serum of TOTP-treated animals detoxified an average of 0.48 μg and 0.2 μg of malaoxon/mg of tissue/10 minutes respectively as compared with 3 μg for liver and 1.3 μg for serum obtained from control animals. The results indicate that TOTP increases susceptibility of rats to malathion by inhibiting the esterases responsible for its detoxification.

Discussion. The present investigation demonstrated that quantities of triorthotolyl phosphate(TOTP), which produce no apparent deleterious effects, markedly increase susceptibility of rats to malathion. Potentiation of toxicity by simultaneous administration of various pairs of organic phosphates including malathion plus EPN and malathion plus dipterex (O,O-dimethyl-1-hydroxy-2,2,2-trichloroethylphosphonate) has been observed by several investigators during past 2 years(1-4). However, in the past the search for agents capable of potentiating toxicity of malathion and other organic phosphates has been limited to testing pairs of compounds used as insecticides. The existing evidence indicates(5,7) that low mammalian toxicity of malathion is highly dependent upon rapid hydrolytic detoxification of the 1,2-dicarbethoxyethyl side chains and that EPN potentiates the toxicity of this compound by inhibiting its detoxification. It thus seemed reasonable to assume that any chemical agent capable of inhibiting hydrolytic detoxification of malathion would potentiate its toxicity. In this connection the known ability of TOTP to inhibit non-specific esterases(8) of liver stimulated our interest in conducting the present investigation. The data obtained during this study demonstrate that toxicity of malathion to rats is markedly enhanced by prior or simultaneous administration of TOTP and that potentiation of toxicity is due to inhibition by TOTP of enzymatic detoxification of malathion. TOTP is effective as a potentiator of toxicity of malathion when it is administered by any route through which a sufficient amount is absorbed to inhibit enzymatic hydrolysis of malathion. The degree of potentiation is greater when TOTP is given prior to malathion so that inhibition of the detoxifying enzyme is maximal before the latter compound is administered. Degree of potentiation obtained with TOTP and malathion was greater than that observed thus far with combinations of any 2 organic phosphorus-containing insecticides which have been studied.

It is clear from our results that the scope of the problem of potentiation of toxicity of organic phosphates extends beyond the possible interference by one insecticidal organic phosphate with metabolism of another compound having a similar chemical structure and use. A complete evaluation of the potential health hazards which might arise as a result of potentiation of toxicity of insecticidal organic phosphates would require consideration of drugs, industrial chemicals, food additives and other chemical agents which commonly gain entrance to the body. The problem is further complicated by the fact that a potentiating agent such as TOTP will inhibit detoxification of malathion at dosage levels which produce no detectable toxic effects. The use of toxicity tests for detection of potentiation by numerous possible combinations of chemical agents which can be ingested simultaneously is not possible from a practical standpoint. However, extension of our present knowledge of the mechanism by which certain compounds act as potentiating agents should greatly facilitate detection of combinations of insecticides and other agents which cause potentiation of toxicity.

Summary. Toxicity of malathion to male rats was markedly increased after treatment of animals with TOTP. At 24 hours after dermal application or intraperitoneal administration of 110 mg/kg of TOTP the LD_{50} of malathion was reduced from normal value of 1100 mg/kg to 12.5 and 8.2 mg/kg respectively. The increased susceptibility of rats to malathion persisted for as long as 14 days after treatment with TOTP. When TOTP was given 24 hours after malathion no potentiation was observed. TOTP inhibited enzymatic detoxification of malathion thus providing an

explanation for its potentiating action. These findings indicate that the problem of potentiation of toxicity of organic phosphate insecticides extends beyond those agents commonly used for insecticidal purposes.

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Temperature Tolerance and Kinetics of Thermal Inactivation in *E. coli communior* Phage of Various Concentrations.* (24669)

G. GUHA (Introduced by A. P. Krueger)
Saha Institute of Nuclear Physics, Calcutta, India

Most studies of thermal inactivation of bacteriophages and of other viruses have indicated that the reaction is exponential over a wide temperature range and follows the first order equation $n/n_o = e^{-kt}$(1-4). This paper describes the thermal inactivation of an *E. coli communior* phage in peptone solution; under certain conditions, the kinetics of its destruction appear to deviate from the above relationship.

Materials and Methods. E. coli communior phage(5) suspended in a peptone solution (Difco Bacto-Peptone, 1%, NaCl, 0.5%, pH 7.6) was treated as follows. One ml aliquots of undiluted phage and of samples diluted 1/100 and 1/1000 in peptone solution were either dried *in vacuo* or stored as liquid preparations. For the inactivation studies, samples were heated in a water bath controlled to ± 0.5°C. The number of particles surviving 30 minutes at a given temperature was determined. In addition, estimates were made of survivors after varying times at different temperatures. The rate constant, K, was calculated from the data. The phage counts were made by the Adams technic(6).

Results. With the dried samples, a linear relationship was found between the logarithm of phage destroyed in 30 minutes and the temperature and this was independent of the initial phage concentration (Fig. 1a). On the other hand, the wet preparations behaved differently. The curves for the inactivation "rate" (logarithm of phage destroyed/30 min) plotted against temperature appeared to show a sigmoidal shape, (Fig. 1b, $1b_1$, $1b_2$). With undiluted phage (Fig. 1b) the change in inactivation rate was exponential between 50° and 75°C. Above this, there was a significant decrease in rate change/degree centigrade. In samples diluted 1/100 and 1/1000 (Fig. $1b_1$ and $1b_2$), the phage was inactivated exponentially between 55°-75°C and 58°-75° C respectively; in each case the rate change reached a plateau beyond 75°C.

The kinetics of heat inactivation in wet (undiluted) and dry conditions were studied by plotting logarithm n/n_o (the fraction of phage survivors), at any given temperature, against time, t, in seconds. In the dry state,

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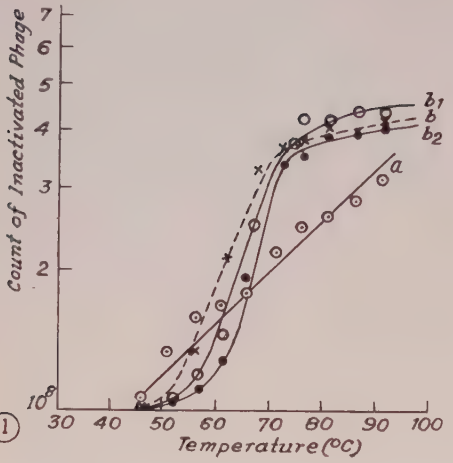
TABLE I. Rate Constants of Dry Phage Samples at Different Temperatures.

Sample	Temp., °C	Time when $n/n_0 = 37\%$ (sec.)	Rate constant $\times 10^4$ (per sec.)
Dry phage	46	4740	2.110
	51	3660	2.732
(Data are same	56	3000	3.330
in all conc. of	61	2223	4.498
dry samples)	66	1659	6.026

inactivation was exponential with time (Fig. 2), but in the wet state, the curve was not linear (Fig. 3). Rate constants for dry samples are shown in Table I. They are independent of initial phage concentration.

Discussion. From the study of thermal inactivation of this coli phage, it appears that phage in the wet state behaves quite differently from phage in the dry state. The cause of this difference is obscure. It is possible that water acts to protect phage particles against the adverse effects of heat. The more dilute the virus preparation, the higher is the temperature at which rate of inactivation begins to vary exponentially with each degree rise in temperature. The reason for the decrease in rate change above 75°C is unknown. If it is assumed that the differences in rate change at the lower temperatures depend solely on initial virus concentration, then the dry samples should be expected to show a similar behavior. Instead, the dry samples exhibit a common inactivation curve regardless of concentration. The data suggest that the effect of environment on reaction rate must be taken into consideration.

Summary. The curves for heat inactivation of an *E. coli communior* phage show that in the dried state, rate of inactivation varies exponentially with each degree rise in temperature, whereas, in the wet state a more complicated relationship exists. This is also reflected by the observation that the dried



①

FIG. 1. Effect of temperature on inactivation of the bacteriophage. (Each point is the mean of ten readings.)

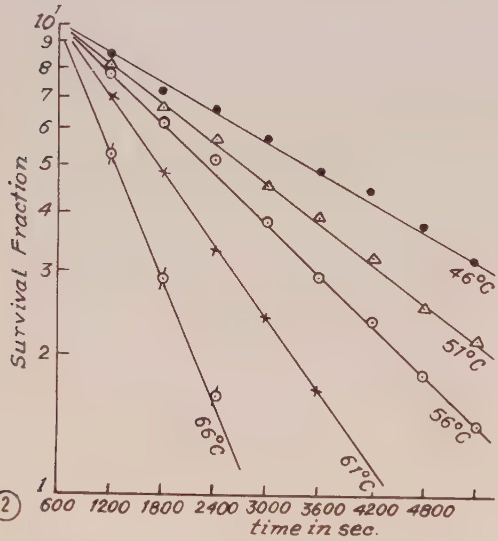


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FIG. 2. Inactivation of dry phages as a function of time at various temperatures. (Each point is the mean of ten readings.)

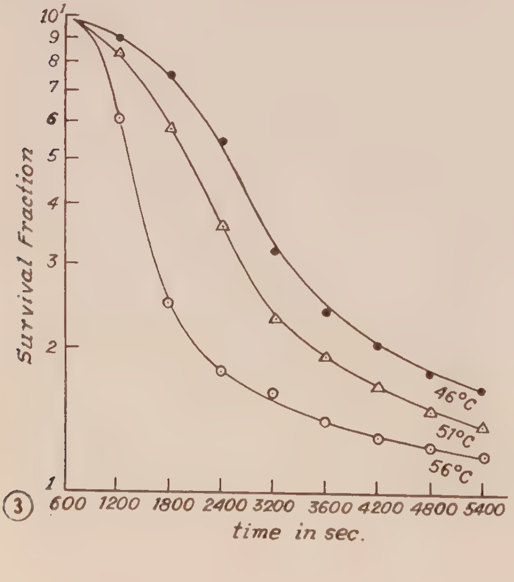

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FIG. 3. Inactivation of bacteriophages in broth (undiluted) as a function of time at various temperatures. (Each point is the mean of ten readings.)

phage is inactivated logarithmically with time over a wide temperature range whereas the wet preparations of phage are not.

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Phospholipids of Human Red Blood Cells.* (24670)

Gerald B. Phillips and Norman S. Roome (Introduced by S. Lieberman)
Depts. of Biochemistry and Medicine, College of Physicians and Surgeons, Columbia University and Presbyterian Hospital, N. Y. City

The wide disagreement in reported values for concentrations of individual phospholipids in human red blood cells(1-5) indicates inadequacy of methods employed. Recently, it was shown that the main phospholipids in serum lipid extracts could be isolated and quantitated by chromatography on silicic acid(6,7). Concentrations of individual serum phospholipids in these studies were at variance with many previously reported values. In the present study, the same chromatographic method was used to isolate and quantitate the main phospholipids of red blood cells of fasting normal human subjects. The main components found were ethanolamine- and serine-containing phospholipid, lecithin, sphingomyelin, and lysolecithin. Concentrations of these components differed from those of several previously reported series(1-4).

Materials and methods. Blood was obtained from 3 male and 3 female apparently healthy young white adults in fasting state and collected in test tube containing heparin. It was centrifuged and plasma and buffy coat removed. Red blood cells were washed 3 times with equal volume of isotonic saline, then packed by centrifugation at 3500 rpm for 30 minutes. One volume of packed red blood cells was pipetted from bottom of tube and hemolyzed with equal volume of water (8). Extraction of this hemolysate was accomplished according to the method of Folch *et al.*(9). The hemolysate was added with shaking to 20 volumes (per volume of packed red blood cells) of chloroform-methanol (2:1, v/v), let stand 30 minutes, and filtered. The red blood cell precipitate was dried, ground to powder, and reextracted with 10 volumes (per volume of packed red blood cells) of chloroform-methanol (2:1, v/v) twice, in the same manner. The 3 filtrates were pooled and then emulsified with 0.2 its volume of 0.05 N KCl solution. The emulsion was broken by centrifugation at 2500 rpm for 15 minutes to give 2 distinct phases without fluff at the interface. The upper phase was removed and the lower phase taken to dryness in rotary vacuum evaporator at maximum temperature of 60°C. This dried extract was stored *in vacuo* at –30° C. Chromatography of the lipid extract on silicic acid columns and on silicic acid-impregnated filter paper and chromatography of acid hydrolysates of phospholipid fractions on filter paper have been described(6,7). Methods used in analyses of column fractions have also been reported(6, 7). In the present study, the standard procedure in normal subjects was to extract 5 ml of packed red blood cells and chromatograph 2 aliquots of the extract, equivalent to 2 ml of packed red blood cells each, on 2 1-g silicic acid columns, respectively.

Results. Three extractions of hemolyzed red blood cells were routinely performed,

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* This investigation was supported in part by research grants from Nat. Heart Inst., P.H.S., and Am. Heart Assn.

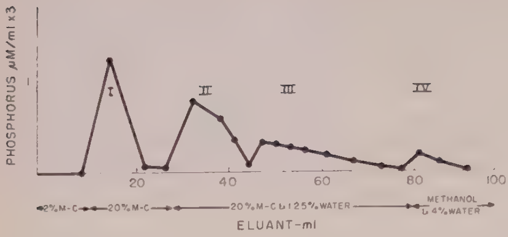

FIG. 1. Chromatography of red blood cell lipid extract on silicic acid (1 g). Predominant peak substances appeared to be: I, ethanolamine- and serine-containing phospholipid; II, lecithin; III, sphingomyelin; IV, lysolecithin.

since the first 2 extractions removed most of the lipid phosphorus, a third extraction about 7-9% in addition, and a fourth extraction about 1-2% in addition. The first 3 extractions, therefore, probably removed more than 95% of total phospholipid extractable under these conditions. Washing pooled extracts with 0.05 N KCl solution removed approximately 10-20% of the extracted phosphorus. This water-soluble phosphorus was assumed to be non-phospholipid phosphorus, as (1) it was insoluble in chloroform and (2) in the test for phosphorus(10) it produced a blue color of similar intensity with or without prior digestion. Phosphorus in the chloroform phase was probably almost all, if not all, present as phospholipid, since (1) in the test for phosphorus it did not react prior to digestion and (2) about 95% of it had the mobility of phospholipid on the silicic acid column.

Chromatography of the lipid extract on silicic acid columns gave rise to 4 peaks of phosphorus concentration (Fig. 1) with mobilities, in order of decreasing Rf, of the "cephalin", lecithin, sphingomyelin and lysolecithin, respectively, of lipid extracts of serum(7). Chromatography of the lipid extract on silicic acid-impregnated filter paper similarly gave rise to 4 spots, the fastest staining with the ninhydrin method, and the 3 slower with the phosphomolybdic acid method. The slowest and faintest spot was streaked down from the origin. Two spots stained with Schiff's stain: (1) the ninhydrin-reacting spot and (2) the fastest spot staining with the phosphomolybdic acid method, the former staining much more intensely than the latter. These 4 spots had mobilities, in order of decreasing Rf, similar to those of the "cephalin", lecithin, sphingomyelin and lysolecithin, respectively, of lipid extracts of serum(7). Hydrolysis of peak materials with 6N HCl at 110°C for 18 hours in a sealed glass tube followed by chromatography on filter paper with butanol-acetic acid-water (4:1:4, using butanol layer) elution showed that the first peak material gave rise to 2 main ninhydrin-reacting spots with mobilities of ethanolamine and serine, respectively, and lesser ninhydrin-reacting spots, but no spot staining with the phosphomolybdic acid method, while the second, third, and fourth peak materials each gave rise to 1 spot staining with the phosphomolybdic acid method, with the mobility of choline, and 2 or 3 minor ninhydrin-reacting constituents. The third peak material (sphingomyelin), in addition, produced a second spot staining with the phosphomolybdic acid method but with an Rf greater than that of choline; a spot with similar mobility and concentration in proportion to the choline spot was seen with the hydrolysate of the third peak material of serum(7). This spot remains unidentified. Phosphorycholine,[†] a possible hydrolytic product of sphingomyelin, did not stain with the phosphomolybdic acid method.

When these acid hydrolysates of peak materials were chromatographed on filter paper using acetone-water (4:1) elution, a spot with the mobility and staining properties of inositol was found only with the first peak material. This inositol, however, was estimated to comprise on a molar basis less than 1% of total red blood cell phospholipids.

The last peak materials of 2 samples were rechromatographed on the silicic acid column using the same solvent system as for the lipid extract. The phosphorus redistributed approximately as follows: "cephalin" 12%, lecithin 16%, sphingomyelin 22%, and lysolecithin 50%. It was assumed, therefore, that about one-half of the last peak material was lysolecithin, and in calculating the relative distribution of individual phospholipids, the other half was added to the other 3 fractions

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[†] Obtained from Mann Research Labs., N. Y., as barium phosphocholine chloride. Sodium salt dissolved in 0.1 N HCl and applied to filter paper likewise did not stain.

TABLE I. Duplicate Analyses of Red Blood Cell Phospholipids of 6 Normal Subjects.

		% total lipid P					
Sex	Age	"Cephalin"*	Lecithin†	Sphingomyelin	Lysolecithin‡	% recovery of P added to column	μMoles lipid P/ml RBC
♂	23	43.0	33.5	21.8	1.7	99	4.80
		42.7	34.7	20.7	1.9	97	
♂	24	42.5	30.0	26.0	1.5	97	4.41
		42.4	29.3	26.7	1.6	99	
♂	30	41.2	36.0	20.5	2.3	101	4.77
		41.7	38.3	18.0	2.1	95	
♀	26	44.6	33.3	20.3	1.8	88	4.44
		41.0	33.1	23.9	2.0	94	
♀	30	40.7	34.3	23.1	1.9	97	5.11
		41.9	32.5	23.9	1.7	94	
♀	31	44.7	29.4	24.0	1.8	96	4.43
		43.8	29.9	24.3	2.0	97	
	Avg	42.5	32.9	22.8	1.9	96	4.66

* Includes plasmalogen and inositol-containing phospholipid.

† Includes plasmalogen.

‡ Estimated as being 50% of Peak IV; 12% of Peak IV added to "cephalin," 16% to lecithin, and 22% to sphingomyelin (see text).

according to the above percentages. The last peak material, both before and after rechromatography, was hemolytic when incubated for 2 hours at 37°C with 2 ml of a 1% suspension of washed sheep red blood cells suspended in isotonic saline; 0.05 μM of the rechromatographed material produced moderate hemolysis (0.01 μM of the corresponding lysolecithin fraction obtained from serum produced barely visible hemolysis(6)). The molar ester bond-to-phosphorus ratio was 1.1 in the one sample analyzed.

The average molar ester bond-to-phosphorus ratio of the "cephalin" peak in the 4 samples analyzed was only 1.2, while this ratio in the one sample of the lecithin peak analyzed was 1.5. A similar ratio in the lecithin peak was found in serum(7).

Analyses for concentrations of individual phospholipids were carried out in duplicate on red blood cells of 6 apparently normal adults in the fasting state (Table I). The valley between lecithin and sphingomyelin peaks was usually not as deep as depicted in Fig. 1; the valley tube was calculated as containing one-half lecithin and one-half sphingomyelin(7).

Discussion. The values found for relative concentrations of individual serum phospholipids were at variance with results obtained by others(1-4) and agreed best with those reported by Hack(4). Previous reports have shown considerable variation between normal subjects in the same series, as well as between series of different investigators(1-4), suggesting inadequacies in methods employed. In contrast, the relatively good agreement between duplicate samples and the rather small variation between subjects in our study provide evidence for reproducibility of the method. The present method, moreover, seemed relatively simpler than previous methods and was carried out on only 2 ml aliquots of packed red blood cells, although a considerably smaller quantity could have been used. At least as much lipid phosphorus appeared to be extracted as with previous methods.

The phospholipid fractions separated in our study were to some extent mixtures. The "cephalin" fraction was composed largely of ethanolamine- and serine-containing phospholipid; in addition, there was evidence for plasmalogen, a small amount of inositol-containing phospholipid, and unidentified ninhydrin-reacting components. Axelrod *et al.*(5) estimated that of total red blood cell phospholipids, 23% was phosphatidyl ethanolamine and 15% phosphatidyl serine. The lecithin fraction apparently contained some plasmalogen and the sphingomyelin fraction an unidentified phospholipid, evidence for which was also found in the sphingomyelin fraction of serum lipid extract(7). The lysolecithin fraction contained material from the other 3 fractions; from rechromatography studies, it was estimated to contain about 50% lysolecithin. Hydrolysates of all fractions contained small amounts of ninhydrin-reacting substances which may have represented constituents of as yet unidentified phospholipids. The low molar ester bond-to-phosphorus ratios of the "cephalin" and lecithin fractions, as was observed with serum (7), may be related to a defect in the ester bond method, to alteration of phospholipids during isolation procedure, or to presence in these fractions of other phospholipids, such as plasmalogen, with ester bond-to-phosphorus ratios of less than 2.

No previous investigators reported the presence of lysolecithin, which comprised about 2% of total red blood cell phospholipids. It is unlikely that this represented adsorbed serum lysolecithin, as the red blood cells were washed 3 times with isotonic saline before extraction, or that it was produced as an artifact during the extraction or isolation procedure(6).

It is of interest to note the striking contrast in the relative proportions of individual phospholipids in red blood cells as compared to serum, especially since there is evidence for exchange of phospholipids between these 2 compartments(11,12).

Summary. Human red blood cells were analyzed for individual phospholipids using chromatography on silicic acid. Concentrations of the main components, which appeared to be ethanolamine- and serine-containing phospholipid, lecithin, sphingomyelin, and lysolecithin, were measured in red blood cells of 6 normal fasting subjects.

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Effect of Epinephrine upon Plasma Optical Density, Unesterified Fatty Acids, Lipemia Clearing and Heparin Levels. (24671)

H. ENGELBERG*
Cedars of Lebanon Hospital, Los Angeles, Calif.

The role of epinephrine in the physiology of fat mobilization has been known, and experimental evidence has been summarized(1,2). Importance of plasma unesterified fatty acids as the major fat transport mechanism involved in this effect of epinephrine was recently revealed(3,4). Subsequently tissue studies indicated that there was increased triglyceride lipolysis in fat depots following adrenalin injection(5), and that unesterified fatty acids were released(6). In these studies no observations were made of plasma lipemia clearing activity or of heparin levels. Since heparin lipoprotein lipase system probably plays a physiologic role in removal of alimentary lipemia from the bloodstream, and perhaps in tissue triglyceride lipolysis, it seemed desirable to study this mechanism in plasma following injection of epinephrine.

Procedures and methods. The hormone was administered intravenously during post-absorptive period, in 9 dogs and 4 human volunteers. The former received .1 - .2 ml of 1:1000

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* Supported by U. S. Public Health Service Grants.

TABLE I. Plasma Optical Density, Lipemia Clearing Activity and Unesterified Fatty Acids before and after Intravenous Epinephrine in 9 Dogs.

	Intrav. epinephrine, .15 ml, 1:1000				
Measurement	Before	2-3′	5′	10′	15-20′
Plasma O.D.	.04	.03	.04	.03	.04
Lipemia clearing*	3	4	4	3	4
Plasma U.F.A.†	1.7	1.7	2.3	2.3	1.8
Plasma O.D.	.02	.04	.02	.03	.02
Lipemia clearing	2	3	3	3	3
Plasma U.F.A.	.9	.9	1.0	.9	.8
Plasma O.D.	.03	.03	.03	.02	.02
Lipemia clearing	1	1	0	1	1
Plasma U.F.A.	1.5	1.3	1.8	1.9	1.3
Plasma O.D.	.03	.04	.02	.03	.02
Lipemia clearing	2	3	1	1	0
Plasma U.F.A.	1.0	1.25	1.45	1.0	.95
Plasma O.D.	.03	.04	.04	.05	.03
Lipemia clearing	3	2	4	3	2
Plasma U.F.A.	1.1	1.15	1.2	1.4	1.0
Plasma O.D.	.03	.03	.03	.03	.05
Lipemia clearing	0	0	1	0	0
Plasma U.F.A.	1.45	1.7	1.9	1.6	1.4
Plasma O.D.	.05	.06	.09	.08	.07
Lipemia clearing	3	3	2	3	3
Plasma U.F.A.	.80	.85	1.05	.95	1.05
Plasma O.D.‡	.07	.21	.19	.16	.12
Lipemia clearing	18	14	20	18	18
Plasma U.F.A.	1.1	1.15	1.15	1.1	.80
Plasma O.D.	.04	.05	.04	.04	.04
Lipemia clearing	8	8	8	9	9
Plasma U.F.A.	1.65	1.9	2.3	1.9	1.8

* Optical density decrease in 1 hr upon incubation of plasma + coconut oil emulsion.

† Values in meq/l.

‡ All blood samples slightly hemolyzed.

adrenalin whereas in humans 1 ml of 1:1000 solution was diluted with 100 ml of physiological saline and given as continuous intravenous infusion for 20 minutes. Venous blood samples were taken prior to and at frequent time intervals after injection, placed in cold citrated tubes, and the plasma immediately separated in refrigerated centrifuge. Analyses were performed soon after plasma was obtained. In all samples plasma optical density, unesterified fatty acids, lipemia clearing activity, and additionally in humans, heparin levels, were determined. Optical density was measured in Coleman Jr. spectrophotometer at 700 mμ. Unesterified fatty acids were extracted and titrated by Borgstrom's method (7). Lipemia clearing activity was determined using 1 ml plasma plus .4 ml of .25% suspension of coconut oil(8). Endogenous plasma heparin was extracted in duplicate and assayed by its anticoagulant activity as previously described(9).

Results. Data obtained in 9 dogs are shown in Table I. Plasma optical density was unchanged in 7 animals, possibly slightly increased in one (#7), and the results were unreliable in #8 because of hemolysis. Lipemia clearing activity, due to serum lipase in the dog and not to lipoprotein lipase, was not altered after epinephrine. In 7 dogs there was a rise in unesterified fatty acids following injection. Results in 4 human subjects are presented in Table II. There was no change in plasma optical density, and no increase (a decrease occurred in one individual) in lipemia clearing activity during infusion for 20 minutes. Unesterfied fatty acids were definitely

TABLE II. Plasma Lipemia Clearing Activity, Heparin Level and Unesterified Fatty Acids after I.V. Adrenalin in 4 Humans.

Sex	Age	Time after adrenalin I.V., min.	Plasma optical density	O.D.* decrease in 1 hr	Plasma heparin	Plasma U.F.A.,† meq/l
♀	27	0	.03	0	13	1.55
		10	"	0	15	1.9
		20	.04	0	16.5	2.5
♀	28	0	.03	6	19	1.45
		10	"	8	18	1.5
		20	.04	6	19	1.95
♂	27	0	.03	18	17.5	1.25
		10	"	7	19	2.05
		20	.04	8	17.5	2.4
♀	20	0	.03	0	17.5	1.3
		10	"	0	20	1.7
		20	"	0	18.2	2.2

* Optical density.

† U.F.A.—Unesterified fatty acids.

elevated in all cases, and there was apparently a slight rise in heparin levels in 3 of the 4 individuals. Although not shown, pulse rate and blood pressure rose moderately in all instances.

Discussion. Plasma optical density changes and those of neutral fat(10) or total lipids and chylomicron counts(11), are usually parallel. Our findings of fairly constant plasma optical density, therefore, indicate that there is no triglyceride rise in plasma after epinephrine during initial period of fat mobilization and plasma unesterfied fatty acid elevation, thus supporting observations(5,6) which showed that fat is released from adipose tissue stores as unesterified fatty acid rather than as triglyceride. Further, failure of plasma lipemia clearing activity to increase after hormone injection, although unesterified fatty acids became elevated, is evidence that the latter did not occur as a result of plasma triglyceride lipolysis. This possible source of increase in plasma unesterified fatty acid was not completely ruled out by previous studies.

The rise in plasma heparin levels, which is certainly not statistically significant but which is borne out by other observations (unpublished) in 2 human volunteers subjected to prolonged stress (72 hours of sleep deprivation) has not previously been noted. It is of interest that there was no concomitant increase of plasma lipemia clearing factor, implying that a dissociation can exist between a stimulus to heparin release and lipoprotein lipase formation. In these experiments it is probable that the rise in heparin was part of a stress reaction, similar to increased fibrinolytic activity in blood after adrenalin(12), rather than a response to increased lipemia (13). Results are at variance with an earlier report(14) of decrease in protamine titre in blood after epinephrine, and illustrate that the protamine titration procedure is not always specific for heparin.

Summary. Immediate effects of intravenous epinephrine in dogs and humans upon plasma optical density, lipemia clearing activity, unesterified fatty acids and heparin levels were determined. There was no change in the first 2 parameters. Unesterified fatty acids were definitely increased in both species. Circulating heparin was increased in 3 of 4 humans studied. These findings indicate that elevation of unesterified fatty acids did not occur as a result of enhanced plasma triglyceride lipolysis, and that the rise in heparin was probably part of a stress reaction unrelated, in this instance, to its role in fat transport.

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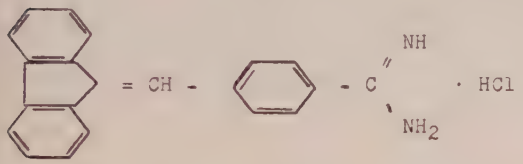
MER-27, a Suppressant of Non-Bacterial Pneumonia in Mice. (24672)

Katherine A. Ludwig, Isaac Ruchman and F. Joseph Murray
Dept. of Microbiology, Wm. S. Merrell Co., Cincinnati, O.

Suppression of non-bacterial pneumonia in mice has been accomplished by use of Xerosin, a crude bacterial filtrate(1). This extract arrested lung consolidation caused by viruses (1-4) and *E. coli* endotoxin(5). In our research program we uncovered a synthetic compound, MER-27, which also arrests lung consolidation caused by viruses or endotoxin.

Materials and methods. MER-27 is chemically 9-(*p*-guanylbenzal)-fluorene hydrochloride.



The drug is insoluble in water and suspensions were made with the aid of a small amount of Triton.[*] *Xerosin.* This was prepared from culture filtrates of *Achromobacter xerosis* n. sp. and was kindly supplied by Dr. Vincent Groupé. Desired concentrations were prepared in water. *Induction of Pneumonia.* a. *Influenza (PR8) Virus.* For viral inoculation 0.05 ml of 10^{-4} dilution of frozen mouse lung suspension containing 10,000 ID_{50} were instilled intranasally into mice under light ether anesthesia. Three days later the mice were killed and at autopsy the degree of consolidation determined, according to the method of Horsfall(6). Values of 0-4+ were given to each lung. Average lesion score of treated mice/average lesion score of untreated controls x 100 yielded the index. Values of 50 or less indicated activity. The lower the figure the greater the activity. b. *E. coli Endotoxin (LPS).* Mice were inoculated intranasally as above with 0.1 mg of LPS[†] and degree of consolidation determined at 3 days. *Swiss mice* of either sex weighing between 10 and 12 g were used for virus inoculation and those weighing between 18 and 20 g were used for LPS inoculation. *Treatment.* Unless mentioned otherwise, aqueous suspensions of drug were administered subcutaneously once a day for 3 days. One hour after first dose, mice received either the influenza virus or LPS. Animals were sacrificed on third day after infection and degree of consolidation compared with that of untreated controls.

Results. Effects of MER-27 on Lung Consolidation Induced by Influenza Virus. a. *Consolidation.* Results in Table I show that at maximum tolerated dose of 50 mg/kg for MER-27 and 100 mg/kg of Xerosin both compounds were equally effective in suppressing pneumonia.

b. *Effectiveness of MER-27 by Various Routes of Inoculation.* Having established that MER-27 was active in suppressing viral pneumonia, the next experiments were concerned with dose-effect relations and route of inoculation (Table II). Confirmation was readily obtained that subcutaneous treatment of mice with MER-27 arrested development of pneumonia. Inhibition was demonstrated with daily doses of 20 mg/kg or more, with borderline activity at 10 mg/kg. Activity was exhibited by the peritoneal route only when the maximum tolerated dose of 20 mg/kg was given. The finding that MER-27 was effective by oral route was of interest. A dose of 100 mg/kg was active and at the 50 mg/kg level there was still some activity. Xerosin was not active orally.

c. *Failure of MER-27 to Influence Viral Multiplication.* It has already been shown that Xerosin interferes with production of pulmonary consolidation without having any effect on multiplication of the virus. Experiments indicated that MER-27 acts in a similar manner. Mice were infected intranasally with serial 10-fold dilutions of virus prepared in phosphate buffer with pH 7.2. One group of mice was treated with MER-27 (40 mg/kg subcutaneously) prior to infec-

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[*] Winthrop Labs. WR-1339.

[†] *E. coli* lipopolysaccharide from Difco Labs.

TABLE I. Effects of MER-27 and Xerosin on Influenzal Pneumonia in Mice.

Drug	mg/kg*	Degree of lung consolidation†																Avg score	Index‡
MER-27	50	3	2	2	1	1	1	1	1	0	0	0	0	0	0	0	0	.7	26
Controls		4	4	4	3	3	3	3	3	3	3	2	2	2	2	1	1	2.6	
Xerosin	100	2	1	1	1	1	1	1	1	0	0	0	0					.8	26
Controls		4	3	3	3	3	3	3	2	2	2	2	1					3.1	

* Daily for 3 days subcut.

† 0-4+ indicates degree of lung involvement.

‡ Index = Avg treated/avg control × 100.

tion, followed by MER-27 inoculations at 24 hours and again at 48 hours. Another group was untreated. Results of titrations in treated and untreated groups showed MER-27 was without effect on the LD_{50} titers of challenged mice. A more definitive study of comparative titrations in treated and untreated mice was instituted. Serial dilutions of virus were instilled intranasally as before. Treated mice were given 5 doses of drug subcutaneously according to following schedule: First dose one hour prior to infection, subsequent doses 24, 30, 48 and 54 hours later. MER-27 at dosage levels employed exerted little effect on LD_{50} titers which were recorded daily. Additionally, at conclusion of test, surviving mice were sacrificed at ninth or eleventh day and degree of lung consolidation calculated to yield ID_{50} (Infective Dose) titers. Again significant differences were not observed, thus indicating that without any effect on virus multiplication MER-27 could still substantially interfere with lung consolidation.

TABLE II. Effects of MER-27 on Influenzal Mouse Lung Consolidation.

Route	Dose,* mg/kg	Avg score Treated	Control	Index
Subcut.	5	2.0	2.8	71
	10	1.6	"	57
	20	.9	"	33
	40	.5	2.4	21
	50	.8	2.5	32
Intraper.	2.5	2.4	2.5	96
	5	2.0	"	80
	10	1.5	"	60
	20	1.3	"	52
Oral	12.5	2.0	2.3	87
	25	2.1	"	91
	50	1.3	"	57
	100	1.0	"	43

* Daily for 3 days.

Effects of MER-27 on Lung Consolidation Induced by LPS. Groupé has shown that LPS administered intranasally produces in mice an inflammatory response in lungs similar to that seen after influenza virus inoculation(5). This type of response was modified by treatment with Xerosin. Results of tests in Table III show that MER-27 suppressed pneumonia induced by LPS.

TABLE III. Effect of MER-27 on Chemically (LPS*) Induced Pneumonia in Mice.

Treatment†		Avg score‡	Index
MER-27	50 mg/kg	.6	25
Controls		2.4	
MER-27	100 "	.4	19
Controls		2.1	

* LPS—10 mg/kg intranasally.

† Treatment—MER-27 subcut. for 3 days.

‡ Score—determined on 3rd day.

Discussion. Suppression of viral lung lesions in mice has been demonstrated following administration of Xerosin, a crude bacterial filtrate(1). Further work showed that Xerosin modified lung lesions which were induced by inoculation of certain chemicals(4,5). Because of its action in suppressing edema, hemorrhage and cellular infiltration in lungs, Xerosin was properly classified as an anti-inflammatory agent. In its action Xerosin closely resembled Cortisone. A comparison between Xerosin and Cortisone showed that although both compounds were effective anti-inflammatory agents, certain differences emerged(5). Xerosin was able to suppress virally and chemically induced pneumonia; Cortisone was effective only against chemically induced pneumonia. Xerosin could modify influenza - precipitated neurotoxicity, whereas Cortisone could not. Another difference is the known ability of Cortisone to enhance infection, whereas Xerosin was unable to do so.

In a search for a chemically defined compound with Xerosin-like activity various synthetics with anti-inflammatory activity were evaluated. MER-27 showed most promise and was extensively investigated. Its activity in suppressing pneumonia more closely paralleled that of Xerosin. However, certain advantages over Xerosin stood out: 1. MER-27 was effective by oral route; Xerosin was not. 2. Xerosin introduced into lungs caused pneumonia which could be arrested by MER-27 or Xerosin itself. MER-27 was unable to cause lung consolidation. 3. MER-27 was a synthetic compound. A recent report(5) that a derived pyrimido-pyrimidine was active in suppressing pneumonia was not borne out in our tests. At most, this compound showed only a borderline activity which according to our criteria would have been regarded as negative.

Our results demonstrate ability of MER-27 to suppress non-bacterial pneumonia in mice. Other activities of this compound will be reported.

Summary. An organic synthetic compound, MER-27, suppresses pneumonia in mice induced by influenza virus when given parenterally or orally. The compound has no effect on viral multiplication. MER-27 also suppresses pneumonia induced by *Escherichia coli* endotoxin. MER-27 does not enhance viral infection and therefore closely resembles Xerosin in its action.

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Nitrous Oxide Solubility in Fetal and Uterine Tissues in Human Pregnancy.* (24673)

N. S. ASSALI AND M. ROSS
Dept. of Obstetrics and Gynecology, University of California Medical Center, Los Angeles

The formula $\left(CBf = \frac{V_u S}{\int_0^t (A-V)\,dt} \right)$ devised by Kety and his co-workers(1) to calculate cerebral blood flow contains in its numerator a factor (S) which refers to the ratio of N^2O dissolved per g of brain to that dissolved per cc of blood at a constant N^2O tension and at 37°. In adopting this formula for measurement of uterine blood flow in human pregnancy(2), the factor S was taken empirically as equal to unity, a value which had been found experimentally for the brain. Others(3) have determined this factor on homogenates of a whole fetus but the age and condition of the fetus were not given and the values for each type of tissue were not known. Because of the variety of tissues contained in a pregnant uterus, it was deemed essential to determine this factor for each individual fetal tissue as well as for the myometrium and placenta. In this way, not only the N^2O technic for measuring blood flow to the pregnant uterus would be better substantiated but also solubility of N^2O in the various fetal tissues would be known.

Method. Samples of fetal tissues varying in weight from 2 to 10 g were obtained immediately after death from one 5 months immature fetus (therapeutic abortion), one 8 months premature fetus and one full term fetus. The latter 2 fetuses died shortly after delivery for no obvious reasons. The tissues studied are listed in Table I. Samples of myometrial tissue were obtained from patients undergoing cesarean section and placental tissues were collected from spontaneously delivered pregnancies. Each sample weighed accurately, homogenized, equilibrated

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* Supported by grants from U.S.P.H.S.

TABLE I. Solubility of N^2O in Human Fetal and Uterine Tissues at 37° and at N^2O Tension of 760 mm Hg.

Tissue	α N^2O (per g tissue)	α Tissue / α Blood
Spleen	.589	1.430
Skel. muscle	.473	1.148
Skin	.458	1.112
Liver	.377	.915
Brain	.439	1.065
Heart	.496	1.204
Lung	.432	1.048
Scalp	.327	.798
Placenta	.331	.803
Umbilical cord	.333	.808
Uterine muscle	.472	1.145
Mean	.430	1.043

with N^2O, and its Bunsen solubility coefficient (α) was calculated according to the technic of Kety *et al.*(1). From 5 to 8 determinations of Bunsen coefficient were made for each type of tissue. Variations from one determination to another did not exceed 1% (±.003).

Results. Table I lists Bunsen solubility coefficients (α) of all fetal tissues studied and of uterine tissue. Placenta, umbilical cord and scalp had the lowest solubility coefficient for N^2O while the spleen had the highest. Average value for solubility coefficients of all the fetal tissues and of uterine muscle was 0.430. There was no significant difference between values obtained from the immature, premature and full term fetuses.

The tissue-blood partition coefficient for N^2O (S factor) varied from 0.798 for the scalp to 1.430 for the spleen, average 1.043.

Discussion. The present data indicate that the N^2O solubility coefficient varies considerably from one fetal tissue to another, but is the same whether the fetus is immature or at full term. It has been stated that N^2O solubility depends a great deal on the fat content of a given tissue(1). Whether the difference observed in the present studies was due to a difference in fat content of the various fetal tissues cannot be stated. At any rate, in considering the pregnant uterus as a whole, and for the purpose of applying the N^2O technic for uterine blood flow measurement, the mean for all the tissues involved is the most important. This mean was 0.430 which gave a partition coefficient for pregnant uterus and blood of 1.043. These figures are very close to those obtained from homogenates of a whole fetus and myometrium, and also close to the value of unity given to factor (S) in study of uterine blood flow. Therefore, the error due to this factor in the previous data (2) is insignificant.

Summary and conclusion. 1) N^2O solubility in fetal tissues was studied in one immature, one premature and one full term fetus. 2) Bunsen coefficient varied from one tissue to another. The mean for all tissues including placenta, cord and myometrium was 0.430. These figures give a N^2O partition coefficient between whole pregnant uterus and blood of 1.043.

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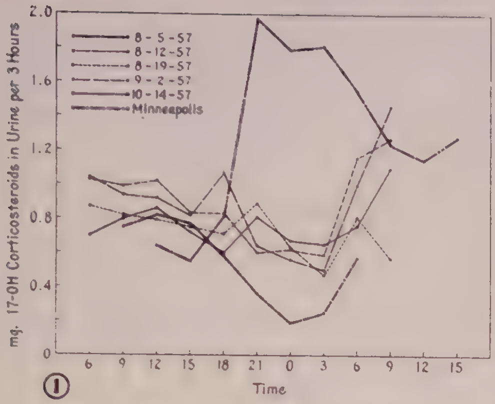
Effect of Sudden Time Displacement by Air Travel on Synchronization of Adrenal Function. (24674)

EDMUND B. FLINK AND RICHARD P. DOE

Veterans Admin. Hospital and Dept. of Internal Medicine, University of Minnesota, Minneapolis

Very few data are available on the effect of sudden change in geographic position on periodicity of physiological functions. Therefore, a study was undertaken on the effect of an airplane trip from Minneapolis, Minn. to Tokyo, Japan, and to Seoul, Korea, with a prolonged stay in Seoul.

Materials and methods. Departure from



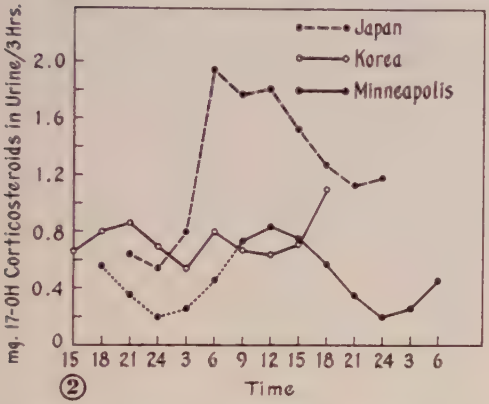


FIG. 1. Times are recorded according to local time. Japan time is displaced 9 hr so peak excretion (21 to 24 hr Japan time) corresponds to 6 to 9 hr Central Standard Time. Korean time is displaced 9½ hr, but excretion is now synchronized with Korean time. Time line indicates time of starting each collection, *e.g.* 6 indicates the period 6:00-9:00, etc.

FIG. 2. Times are recorded according to Central Standard Time. Minneapolis curve has been extended by dotted line to show relationship to Korean and Japanese curves. Korean curve is the one dated Oct. 14 in Fig. 1.

Minneapolis occurred Aug. 1, 1957, from Seattle, Wash. Aug. 3, 1957. Arrival in Tokyo, Japan was Aug. 5, 1957 (Aug. 4 Central Standard Time). Urine collections every 3 hours were started immediately and continued 30 hours. Dates of subsequent collections in Seoul, Korea began Aug. 12, Aug. 19, Sept. 2, and Oct. 14. Urine volumes were measured for each 3 hour period day and night and a portion placed in a test tube with a few drops of toluene as preservative. Three meals composed of usual American foods were eaten at usual meal times. Fluid was not restricted. In a previous study in Minneapolis, however, equal feedings were eaten every 3 hours for 24 hours(1). Specimens were sent to Minneapolis by airmail and analyzed for sodium and potassium by flame photometry and for total 17-hydroxycorticosteroids by the method of Silber and Porter using glucuronidase hydrolysis(2).

Results. Results of 17-hydroxycorticosteroid excretion are recorded in Fig. 1. Potassium and sodium data are recorded in Tables I and II. It is clear that steroid excretion was greatly altered the first 24 hours after leaving United States. The time displacement in Japan is 9 hours and in Korea 9½ hours. Peak excretion occurred at night instead of the day and corresponds quite well with the expected peak for Central Standard Time (CST) (USA). When time is 6:00 in Minneapolis it is 21:00 in Tokyo and 20:30 in Korea. Peak excretion in Japan occurred at 21:00 to 24:00 period which corresponds to 6:00 to 9:00 CST. Lowest excretion in Japan occurred at 15:00 to 18:00 (24:00 to 3:00 CST). Curves in Fig. 2 are plotted so that they all referred to CST instead of local time. Then the excretion curve of 17-hydroxycorticosteroids in Japan superimposes on that in Minneapolis but a representative curve from the Korean study is displaced by 9 hours. The peak was exaggerated though, perhaps because of excitement and anxiety. Subjective symptoms on day of arrival in Japan included extreme sleepiness from 15:00 to 22:00 o'clock even though sleep had been adequate on the plane, and wakefulness from midnight to 6:00. During adjustment period in Korea wakefulness at night and sleepiness in afternoons persisted for about 3 weeks. This was not a daily phenomenon after the first few days and gradually disappeared.

Potassium excretion (Table I) follows the peaks of 17-hydroxycorticosteroid excretion reasonably well. Note the very high excretion during the night of the first 24 hours in Tokyo. The excretion pattern which most closely mimics that in Minneapolis is the one on Oct. 14, 1957. The variation in potassium excretion cannot be accounted for by the fact that food was eaten only during the day. A very

TABLE I. Potassium Excretion, meq/3 Hr.

Date	6–9	9–12	12–15	15–18	18–21	21–24	0–3	3–6	6–9	9–12
8- 5			13.4 5.1	15.0 3.5	5.8	11.4	21.8	17.0	14.9	6.5
12	4.7	5.7	3.6	5.1	5.1	6.7	3.9	4.1	4.2	8.0
19	8.7	7.9	9.3	10.4	8.5	4.4	10.8	4.6	3.7	5.8
9- 2	9.1	11.5	13.8	12.3	9.2	4.3	5.4	3.9	4.3	9.5
10-14	6.0	12.6	12.3	12.3	7.5	4.6	4.5	7.4	7.7	12.7
Minneapolis		15.8	23.6	6.5	8.8	5.7	3.1	3.1	3.7	

Time is recorded as local time.

TABLE II. Sodium Excretion, meq/3 Hr.

Date	6–9	9–12	12–15	15–18	18–21	21–24	0–3	3–6	6–9	9–12
8- 5			18.4 7.2	31.8 9.0	20.8	22.0	37.1	35.8	27.6	13.0
12	12.6	23.0	27.0	25.8	42.1	39.9	25.9	14.4	17.5	13.5
19	13.7	17.8	23.8	21.3	28.4	18.3	25.5	14.9	11.3	33.1
9- 2	4.9	20.4	26.2	25.5	38.8	23.0	22.4	18.6	11.9	23.2
10-14	15.4	34.2	21.7	21.9	24.9	24.9	8.1	21.2	10.3	20.3
Minneapolis	12.2	12.3	11.2	6.6	11.0	16.2	11.0	14.2		

Time is recorded as local time.

large excretion occurred during the night in Tokyo and excretion of potassium in Korea followed quite closely the steroid excretion and was not greatest at the time following largest or evening meal. This becomes even more significant when compared with the excretion study in Minneapolis during which time equal feedings were eaten every 3 hours throughout the 24 hour period. Sodium excretion (Table II) is quite variable. However, there is a marked sodium excretion the first night in Tokyo. The peak of sodium excretion follows the steroid excretion peak very well and corresponds to a group of subjects studied previously(1) but not to the traveler's own pattern in Minneapolis. Sodium excretion is erratic except for the Tokyo study, so the significance of the variations cannot be evaluated.

It is clear that 17-hydroxycorticosteroid excretion reverted to a pattern synchronized with Korean time with displacement of the peak of excretion by approximately 9 hours from CST (USA). This occurred by 11 days after leaving Minneapolis (9 days after leaving Seattle). The striking difference between the first study and the others is apparent at a glance. The amplitude of variation and particularly the decrease at night on all subsequent curves is not as great as one in Minneapolis. There is a small secondary peak of excretion at either the 18 to 21 or 21 to 24 hour period in all but one of the curves during the Korean sojourn.

Discussion. Mammals can adapt or their rhythm becomes synchronized only slowly when subjected to sudden changes in environmental routine. Halberg(3,4) has found that it takes one week or longer for mice to resynchronize the daily rhythm in blood eosinophils with an abruptly inverted lighting schedule. When mice are kept under otherwise controlled conditions significant shifts in phase have been established by 2 weeks after lighting inversion for diverse daily periodic functions, the daily rhythms of mitoses in pinnal epidermis or liver, those of hepatic nucleic acid metabolism and the rhythm of corticosterone in blood are but some of the cases in point(5). In turning to data on human beings, Burton has twice studied his temperature rhythm, when flying from London, Ontario, across the Atlantic to England. He thus altered his routine of living by a phase shift of 5 hours and found that it took 3 to 4 days for his Canadian temperature rhythm to fall in step with the English rhythm (6).

The data of this paper, in turn, show that in man excretory patterns related to cortical adrenal function also can be adjusted to a new schedule of lighting and activity, in 9 days at least. In good agreement with the results obtained on experimental animals it is also apparent that there is not a complete adjustment immediately following a change in routine. The synchronization with a new time schedule also is apparent in the electrolyte excretion.

Summary. 1) Excretion of 17-hydroxycorticosteroids in the urine was measured at frequent intervals throughout 30 hours at 5 different times following a shift from CST to Japanese and Korean time. The excretion was synchronized with CST immediately after arrival in Japan, but became synchronized with the new time schedule by 9 days. 2) Sodium and potassium excretion patterns are similar to that of a previous study on Central Standard Time but synchronized with the new time schedule also in 9 days.

The authors wish to acknowledge help and criticism of Dr. Franz Halberg.

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Wound Healing: Glycoproteins of Wound Tissue. I. Studies of Hexosamine, Hexose, and Uronic Acids Content.* (24675)

M. R. SHETLAR, EUGENIA G. LACEFIELD, BETTY N. WHITE AND JOHN A. SCHILLING
Depts. of Surgery and Biochemistry, University of Oklahoma School of Medicine, Oklahoma City

The importance of connective tissue in wound healing is well recognized. However, little is known about the complicated sequence of events by which connective tissue is formed. Kao, Boucek, and Noble(1) recently reported studies of connective tissue formed following implantation of polyvinyl sponge. Robertson, *et al.*(2) found that following injection of carrageenin suspension into scorbutic guinea pigs, granulomatous repair tissue contained more hexosamine containing polysaccharide material than that formed under the same conditions by normal animals. Slack(3), using a similar suspension, found that increase in polysaccharide material in scorbutic guinea pig tissue was due to increase of hyaluronic-acid-like material, but that chondroitin sulfate decreased. Schilling *et al.*(4) have described a technic for study of wound fluid utilizing stainless steel cylinders which are implanted subcutaneously in animals. This procedure allows removal of numerous samples at various time intervals for studies of tissue and fluid components. The following describes serial studies of hexosamine, hexose, and uronic acid contents of wound tissue formed around such cylinders.

Methods. Stainless steel cylinders were implanted subcutaneously in dogs as previously described for guinea pigs(4). The cylinders used were of #46 mesh and were 1 cm in diameter by 5.5 cm long. Two lucite plastic rings 9.5 mm in diameter and approximately 3 mm thick were placed in each cylinder to keep it from collapsing during the healing process. At stated intervals the cylinders were removed, the fluid within cylinder was drawn off, and extraneous tissue was stripped from the outside of cylinder. The cylinder was opened by longitudinal scissor cut, and the tissue was lyophilyzed still attached to the cylinder. The dry tissue was stripped by hand from the stainless steel mesh and extracted in Soxhlet extractor for 24 hours with diethyl ether to remove lipid material. The extracted tissues were ground to pass through

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* Supported in part by grant from U. S. Army.

TABLE I. Uronic Acid, Hexosamine, and Hexose Contents* of Wound Repair Tissue.

Days after implantation	Avg wt of tissue, mg	Uronic acid	Hexosamine	Hexose
5	200	.57 ±.06	5.79 ±.12	20.9 ± .2
7	157	.72 ±.14	6.80 ±.10	19.4 ± .7
14	221	1.04 ±.14	7.07 ±.25	26.0 ±1.7
21	238	1.37 ±.21	6.19 ±.25	27.4 ±2.0
28	683	1.77 ±.15	7.60 ±.36	22.3 ± .3
28	316	2.35 ±.17	8.47 ±.16	30.3 ± .3
42	402	2.27 ±.09	7.32 ±.23	29.7 ±1.0
56	392	2.80 ±.33	6.73 ±.17	26.6 ± .7

* All results are based on analyses of 6 separate samples and calculated as mg/g of dry lipid-free material. Figures following ± sign are stand. errors.

a 60 mesh screen. Aliquots of ground material were used for chemical analyses. Uronic acid was determined by the carbazole method (5) following preliminary hydrolysis with Dowex 50 resin as previously described(6). A correction for the reaction of hexose with carbazole was made by determining hexose in each Dowex hydrolysate. Under conditions of carbazole reaction, 100 mg of hexose was equivalent to 7 mg of uronic acid. Hexosamine was determined by method modified from that of Boas(7). Hexose was determined by an anthrone method.

Results. Results of analytical studies are presented in Table I. It is readily apparent that the uronic acid (expressed as glucuronic acid) is relatively low at 7 days, increases to maximum level at about 28 days and remains relatively constant thereafter for at least 42 days. On the other hand, hexosamine and hexose levels are already comparatively high at 7 days and do not increase strikingly in later samples.

The definitely higher levels of hexosamine as compared to uronic acid (as determined by the carbazole reaction) levels are of interest. It appears that the polysaccharides which do not contain uronic acid are present in much greater amounts in wound healing tissue than are uronic acid containing mucopolysaccharides. This is particularly true of the early tissue. As verification of these data, preliminary studies of constituent monosaccharides of hydrolysates of wound tissue were performed. Wound tissue was hydrolyzed using Dowex 50 W × 8 as catalyst at 97-100° C. Under these conditions, 75-95% of the glucuronic acid of chondroitin sulfate was still present. These hydrolysates were concentrated under vacuum and subjected to paper electrophoresis in borate buffer. Definite, but weak, bands characteristic of uronic acid were found as compared to relatively strong bands of glucose, mannose, and galactose (Fig. 1). The uronic acid band was decidedly stronger in later sample. All or most of the glucose in the hydrolysates may be assumed present in the tissue as glycogen; however, galactose and mannose must be constituents of polysaccharides or glycoproteins which have not yet been identified.

Summary. A serial study of uronic acid, hexosamine, and hexose content of wound repair tissue was made. Uronic acid content was quite low in the 7-day sample, but increased until about 28 days. Hexosamine in considerable excess of uronic acid was found indicating presence of polysaccharides or glycoproteins which did not contain uronic acid.

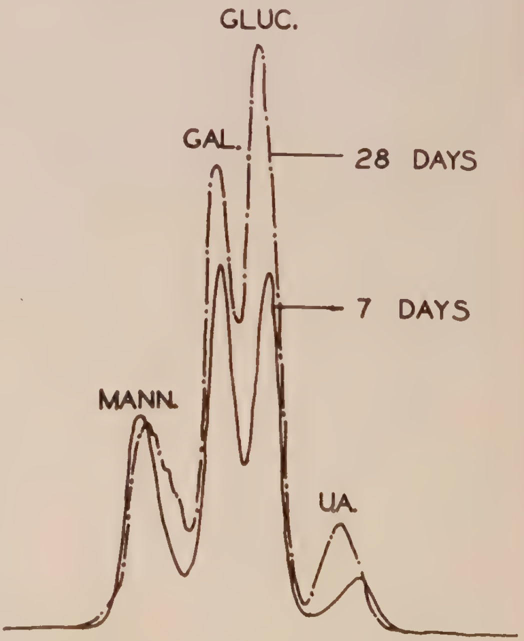

FIG. 1. Densitometer tracings of paper electrophoretic patterns of sugars in hydrolysates from wound repair tissue. Borate buffer .05 M, pH 9.2. Solid line, tissue at 7 days; broken line, tissue at 28 days. Peaks typical of mannose are indicated by "Mann."; galactose by "Gal."; glucose by "Gluc."; and uronic acid by "U.A.". Note increase in uronic acid in 28 day sample.

Sugars with electrophoretic mobility in borate buffer of galactose, mannose, and glucose were identified in hydrolysates of wound repair tissue.

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Acute Effect of Psychologic Stimuli upon Plasma Non-Esterified Fatty Acid Level.* (24676)

MORTON D. BOGDONOFF, E. HARVEY ESTES, JR. AND DAVID TROUT
(Introduced by E. A. Stead)
Dept. of Medicine, Duke University Medical Center, and Vet. Admin. Hospital, Durham, N. C.

Several recent studies have pointed up the important role of non-esterified fatty acids (NEFA) as major transport form of plasma lipid(1,2,3). This segment of plasma lipid undergoes rapid turnover (half-life of approximately 2 minutes)(3), and is promptly altered by administration of such substances as glucose(1,2,6), glucagon(5), insulin(4.6), epinephrine(1,2), and heparin(2). Lability of this lipid fraction provided an opportunity to study the relationship of fat metabolism to acute alterations in affect state within the structure of a short-term, controlled experiment. Other serum lipid fractions varied in response to changes in life-situational patterns, but these studies were usually conducted over long periods of time(7,8,9). The results herein reported clearly indicate that acute alterations in emotional arousal had a distinct effect upon fat homeostasis.

Methods and procedures. Four volunteer subjects (medical students) were each asked to report in early morning in fasting state. They were informed that only a single needle was to be inserted and that samples were to be drawn during sleeping and waking periods during the morning. #18 Cournand needle was placed in antecubital vein under procaine anaesthesia, and the first sample drawn about 10 minutes later. The room was darkened; the subject was asked to relax completely and to sleep if possible. Samples were drawn at approximately 30 minute intervals for one to 2 hours. Then the lights were turned on and a non-directed, light conversation was carried on between various persons. After 15 to 30 minutes, blood was again withdrawn, and all experimenters except one, excused themselves for various reasons. The remaining experimenter engaged the subject in casual conversation, which was quickly focused upon areas of personal conflict, anxiety, anger, and hostility, uncovered during the interview. All subjects became more or less heatedly involved in conversation, all becoming restless, showing changes in facial expression and some changes in skin color. Samples were obtained during this period by another member of the experimental team, who entered only for the period required for obtaining the sample. The conversation was terminated by reentry of other members of the team, who continued with a superficial non-directed conversation for another 15 to 30 minutes, after which the final sample was drawn and the needle withdrawn. Samples were withdrawn into heparinized syringes, which were placed in ice and immediately taken to the analytical laboratory where centrifugation was quickly carried out and the plasma measured into the extraction mixture. NEFA determinations were carried out by the

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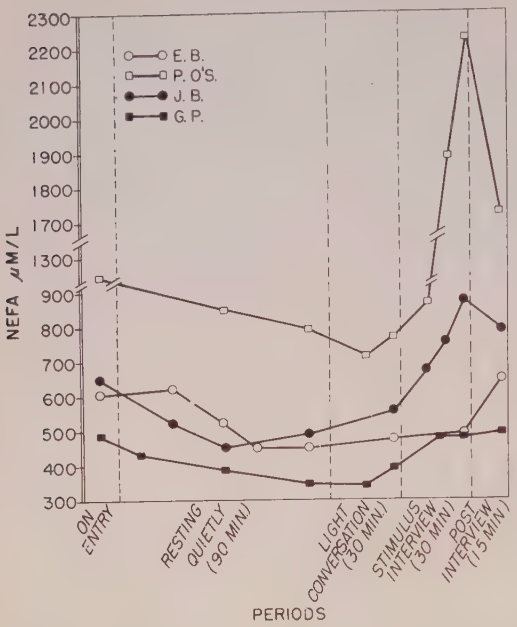

FIG. 1. Time course of serum NEFA values in 4 subjects during sequential periods of various degrees of arousal.

technic of Dole (This method is accurate to ± 20 μeq/l)(1). Triglyceride determinations were done by the technic of Van Handel and Zilversmit(10).

Results. The results are graphically presented in Fig. 1.

Comments. The experiments demonstrate a pattern of response of plasma NEFA levels which relates to degree of emotional arousal. Level of NEFA value is roughly proportional to intensity of emotional arousal and occurs promptly with a change in affect state. These results seem to indicate that fat metabolism is intimately involved in the individual's acute responses to "stressful" stimuli. NEFA is known to be an extremely active transport form of fat(1), and there is evidence that this fraction can be directly utilized by muscle and by liver(2). It is believed to derive from adipose tissue depots. Observations by Dole (1) and Gordon(2) have shown that epinephrine causes a sharp rise in NEFA level. Our observations confirm these data, and also demonstrate that norepinephrine is capable of inducing a similar rise in NEFA levels in human subjects (unpublished). It is our thesis that during emotional arousal these hormones are released and may provide the mechanism for increasing the serum NEFA level. The precise quality of affect experienced during period of emotional arousal did not appear to be the same for all subjects: P.O.'S. and J.B. reported predominant anxiety, E.B. and G.P. less anxiety and some hostility. In all subjects, meaningful topics of both qualities were discussed.

Summary. 1. Serum non-esterified fatty acids (NEFA) and triglyceride (TG) levels were measured in four normal individuals during difficult levels of emotional arousal. 2. Periods of arousal in which feelings of anxiety and/or hostility were evoked were accompanied by rises in serum NEFA levels, but not of TG levels.

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Effect of Aging and Rotation on Human Amnion Cell Response to Polio and Sindbis Viruses.* (24677)

Thomas E. Frothingham† (Introduced by J. F. Enders)
Research Division of Infectious Diseases, Children's Medical Center, Boston, Mass.

Enders(1) noted that the strain of Type II poliovirus adapted to the chick embryo by Roca-Garcia and co-workers(2) was, like other strains, regularly and markedly cytopathogenic for human amnion cells. However, in contrast to polioviruses so far examined, it failed to induce obvious changes in human renal cells. In our initial attempts to repeat Enders' observations, the response of amnion cells to infection proved highly variable. Frequently the cells remained unaffected even when large amounts of this virus were added, although occasionally they were partially or completely destroyed. Comparable irregularities were also seen in cultures of human amnion cells exposed to Sindbis virus, an agent that is highly destructive to chick embryo cells(3). Consideration of differences in techniques employed suggested that rotation of the culture and its age might be important factors in determining response of human amnion cells to these agents. Accordingly, the effect of agitation and aging has been investigated and the results are here summarized.

Materials and methods. Cultures. Primary cultures of human amnion and chick embryo cells were prepared from trypsinized tissues(4,5). The cells were grown on glass in pyrex-brand, stoppered 15 × 150 mm tubes or 160 ml Breed milk dilution bottles and incubated at 35-37°C either in stationary position or in roller wheel operating at approximately 20 rph. Amnion cell cultures were at first nourished with bovine amniotic fluid medium containing 20% horse serum. After inoculation with virus they were maintained wtih bovine amniotic medium (BAF) containing 5% horse serum(4). To establish sheets of chick cells suitable for plaquing (6) the yield from five 10-day embryos was suspended in 200 ml BAF medium and dispensed in 10 ml aliquots into bottles. Fluid was changed 1 or 2 days later and virus added on the 3rd day. At beginning of investigations, Dr. T. W. Chang of New England Medical Center informed us of a possible correlation between sex of conceptus and quality of amnion cultures and cell-reaction to infection. With this in mind the following observations were made. Cultures were prepared with cells from either 1 or 2 membranes. Thirty-eight consecutive lots of cells were used over 9-month period and the cultures were rated as "excellent", "fair", "poor", or "unusable", according to degree of cellular granularity, non-specific degeneration, and presence or absence of extracellular precipitate. Thirteen of 21 lots from female membranes and 2 of 17 lots from male membranes were rated "excellent". Two male lots were unusable and the remaining 21, although rated "fair" to "poor", were nevertheless adequate for work with viruses of known, pronounced cytopathogenicity. Response to viral infection of amnion cell cultures prepared from conceptus of different sex was also investigated. In this respect no consistent differences were observed. *Viruses.* Brunhilde and Lansing strains of poliovirus(7) were derived from 3rd and 4th human tissue culture passages. Rogers strain of Type II poliovirus was isolated by Dr. Sidney Kibrick from stool obtained at post mortem examination of patient with fulminating bulbo-spinal poliomyelitis. Material from third human tissue culture passage was used. Chick embryo adapted Type II MEF$_1$ poliovirus(2) was obtained through kindness of Dr. Herald Cox of Lederle Labs. in the form of material from 140th egg passage. It was passed once in eggs in this laboratory and once in human amnion cell cultures. Materials from both the egg passage and tissue culture passage were used. Types I, II, and III attenuated polioviruses currently under test as vaccines

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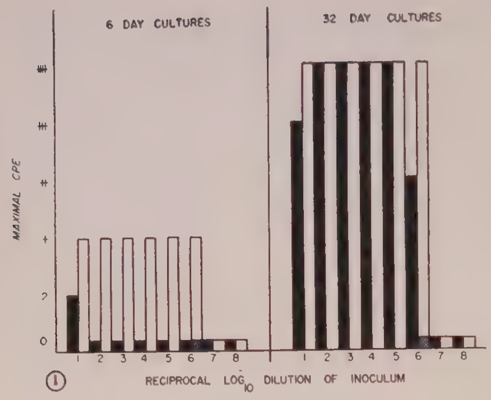
† Fellow of the National Fn.



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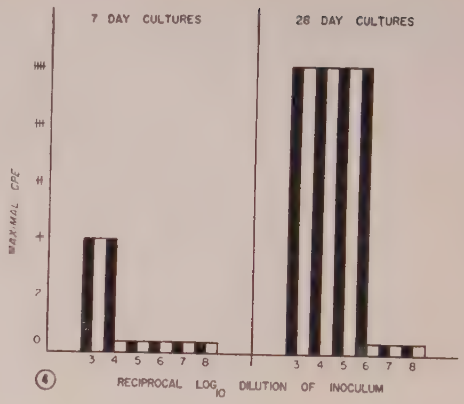

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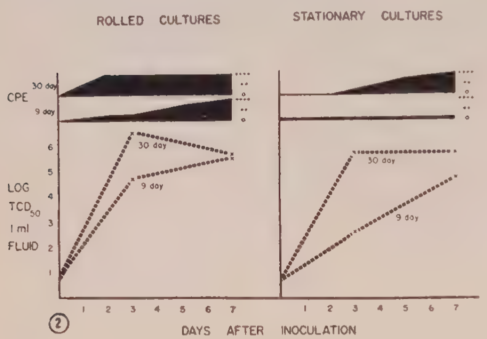

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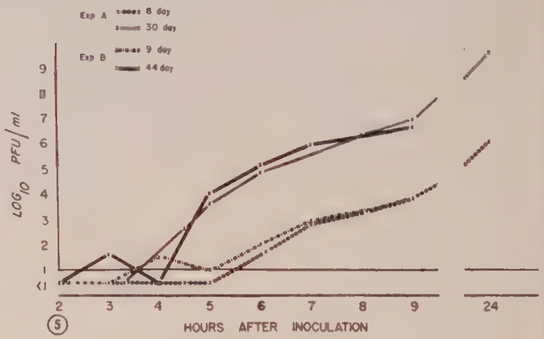

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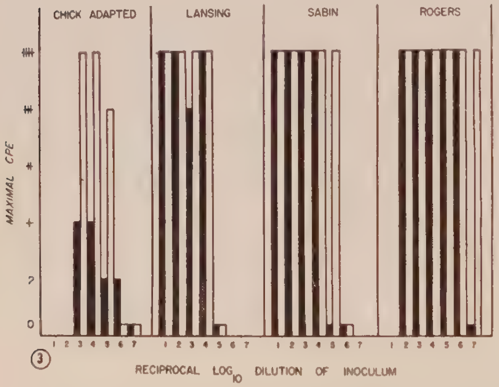

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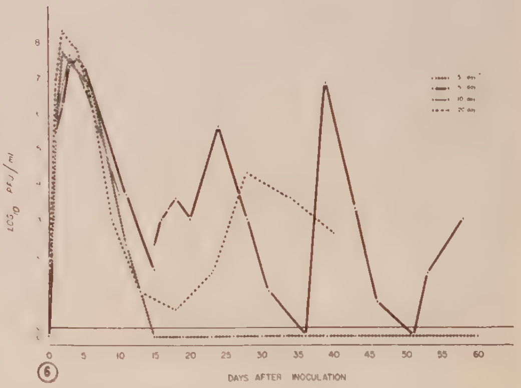

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FIG. 1. Cytopathic response to chick-adapted poliovirus in stationary and rolled human amnion cell cultures of different ages.

CPE–0 — No CPE
? — Minimal degeneration of questionable specificity
+ — Definite foci of degeneration
++ — Degeneration of about 50% cell population
+++ — Degeneration almost complete
++++ — Complete degeneration

Each bar indicates CPE in 3 cultures; black bars, stationary cultures; white bars, rolled cultures; cross-hatching, CPE in 1 of the cultures.

FIG. 2. Cytopathogenicity and multiplication of chick-adapted poliovirus in stationary and rolled human amnion cell cultures of different ages.

FIG. 3. Effect of rolling on the cytopathic response of 14 day human amnion cell cultures to strains of Type II poliovirus. Each bar indicates CPE in 2 cultures. See Fig. 1 for other notation.

FIG. 4. Cytopathic response to Sindbis virus in stationary and rolled human amnion cell cultures of different ages. Each bar indicates CPE in 2 cultures. See Fig. 1 for other notation.

by Dr. Albert B. Sabin, were obtained from him and designated as "Sabin strains"(8). Sindbis virus(9) strain AR339 was furnished by Dr. Richard M. Taylor in the form of 10% suspension of mouse brain. Throughout, material from 10th chick embryo passage was used. Stock viruses were kept in CO_2 cabinet with the exception of Sabin's strains which were stored at –20°C. *Antiserum.* Anti-Sindbis virus rabbit serum was prepared essentially by the method described by Ramos-Alvarez and Sabin for ECHO viruses(10). Virus employed as antigen was grown in chick embryo cultures maintained in medium 199. *Assay of virus.* Serial 10-fold dilutions were prepared in BAF medium. Three tube cultures were inoculated with 0.1 ml of each dilution tested. Titers are expressed as log TCD_{50}/ml of culture fluid. Plaque assay of Sindbis virus in chick embryo bottle cultures was performed essentially by the method of Dulbecco and Vogt as adapted to bottles by Hsiung and Melnick(11). Plaques appeared within 24 hours and attained maximal numbers by 4 days. Titer was expressed as plaque-forming units (PFU)/ml. Assays of stock virus performed on 12 separate occasions during 6 months gave a range of 15-46 $\times 10^8$ PFU/ml with mean of 30×10^8 PFU/ml. *Age of cultures.* This was expressed in days from time of dispensing the cells into culture vessels. Between this procedure and obstetrical delivery 2-5 additional days elapsed. Cultures 5-10 days old were designated as "young," those 11-21 days as "intermediate" and those over 21 days old as "old". *Cytopathogenic effect (CPE).* Unless otherwise specified this was evaluated by frequent low power microscopic inspection during at least 2 weeks after inoculation for poliovirus and 10 days for Sindbis virus.

Experimental. Chick embryo-adapted poliovirus. Cultures were divided into 2 groups. Fluids of the first, designated "young" cultures, were changed on days 5 and 6. After change on day 6, 0.1 ml of a wide range of 10-fold dilutions of virus (10^{-1}-10^{-8}) were added to 6 cultures/dilution. Three of these cultures were then rolled and 3 left stationary. Estimations of CPE were made every 1 to 5 days during following 19 days. Fluids of second group, designated "old" cultures, were changed on days 5, 11, 19, 26 and 32. After last change, cultures were inoculated with dilutions of virus and maintained under same conditions as those in Group 1. Fig. 1 graphically records the occurrence of CPE. It is evident that CPE was minimal or absent in young cultures depending upon whether they were rolled or kept stationary. In old cultures irrespective of motion, the virus rapidly destroyed all or nearly all cells. This experiment was repeated with cells from different amnions, once with the same range of viral inocula and once with single small inoculum. Comparable results were obtained. It seemed possible that the greater number of fluid changes undergone by second group of cultures might be responsible for the difference in response. Accordingly, fluids of 6 cultures of intermediate age prepared from a single lot of cells were changed on days 6 and 13. In addition, fluids of 3 cultures were changed daily on days 8 through 12. Each culture was inoculated with approximately 100 TCD_{50}/ml of virus on day 13 and subsequently incubated in stationary position. No significant difference was noted in CPE which was recorded as "1+" in all.

The effect of age was also studied in cultures prepared with different cell lots but nourished with same lot of medium. Fluids of 8-day and 29-day-old cultures were replaced with the same lot of BAF medium and inoculated with approximately 1000 TCD_{50}/ml of virus. All cultures were incubated in stationary position. The 8-day cultures showed no definite degeneration during ensuing 25 days. In contrast, the 29-day cultures degenerated within 4 days.

Increase of virus and metabolic activity of cells as indicated by rate of pH fall in young and old cultures, were compared as follows. Two groups of cultures derived from a single

FIG. 5. Increase of Sindbis virus in fluid phase of amnion cell cultures of different ages.

FIG. 6. Concentration of Sindbis virus in fluids of 4 amnion cultures in the absence of CPE. +, Age of culture at inoc. ++, This culture reinoc. and washed on day 15. +++, Less than 10 PFU/ml. ++++, No PFU/ml undiluted fluid.

cell lot were inoculated with small dose of virus on 9th and 30th day respectively. Cultures inoculated on 30th day received one additional change of fluid prior to inoculation; otherwise conditions of maintenance were the same. Six inoculated and 6 control tubes from each group were rolled and equal numbers left stationary. CPE and pH as colorimetrically determined were recorded daily. At 3 and 7 days after inoculation fluids from 3 tubes from each of the rolled and stationary sub-groups were pooled and stored. Later, all stored harvests and samples of viral inoculum, which had been stored at –20°C, were assayed simultaneously in 15-day rolled cultures. Initial pH and pH drop in control cultures and in inoculated cultures prior to degeneration were comparable, regardless of age or agitation. As in the previous experiment, results (Fig. 2) indicated that while effect of aging was evident in both stationary and rolled cultures, it was most pronounced in the former. Viral multiplication occurred under all these conditions but in young cultures it was slower. The enhancing effect of rolling on CPE was greater in cultures of intermediate age (Fig. 1, 3).

Unsuccessful attempts were made to demonstrate factors in the fluid phase of young and old cultures that might be responsible for the observed phenomena. Thus BAF medium that had been in contact with incubated young cultures or fresh amniotic membrane at room temperature for at least 24 hours, failed to influence the response of old cultures to virus. Supernatant fluid from fresh amniotic membrane ground in medium also proved ineffective Conversely, medium from old cultures did not increase reactivity of young cultures. It was also determined that susceptibility of stationary cultures of intermediate age was not altered by rotation for 3 days prior to inoculation of the virus. Similarly response of cultures of intermediate age remained unaffected when they were inoculated with virus rolled for 2 hours, then kept stationary.

Experiments with other strains of poliovirus. It seemed possible that cytopathogenicity of other attenuated strains of poliovirus might be similarly influenced by age and rotation of culture. Accordingly 14-day cultures from a single cell lot were inoculated with chick-adapted and Sabin's Type II attenuated strains. Virulent Lansing and Rogers viruses were also included for comparison. Equal numbers of cultures inoculated with each of these agents were rolled and left stationary. Only the cytopathogenic effect of chick-adapted virus was significantly increased by rolling (Fig. 3). Other experiments of this sort were done in which Brunhilde, Lansing and Sabin's attenuated Type I, II and III strains were employed. Rolling failed to enhance significantly the cytopathogenicity of these viruses with the possible exception of Type III strain. In this case a difference was apparent with the smaller inocula.

Sindbis virus. Comparable experiments were carried out with Sindbis virus in which effects of age and rotation of cultures were determined. In Fig. 4 results of one experiment are presented in which cultures prepared from the same lot of cells and maintained with a single batch of medium were employed. It is evident that CPE was markedly enhanced in older cultures and this difference was apparent in all experiments. However, in young cultures, degree of CPE tended to vary more widely than in stationary cultures infected with chick adapted poliovirus. Indeed, in some young cultures complete degeneration of cells occurred. Rolling of cultures infected with Sindbis virus did not alter intensity of CPE (Fig. 4). In this respect Sindbis virus contrasts sharply with chick-adapted poliovirus. Multiplication of Sindbis virus was determined in bottle culture of varying ages by repeated titrations of aliquots of the fluid. A young and an old culture were selected which were estimated by microscopic examination to contain approximately the same number of cells. Inoculum consisted of approximately 3×10^6 chick cell plaque forming units in 1 ml of BAF medium. After an hour's incubation the inoculum was removed and the culture washed 10 times with 10 ml Hanks' salt solution. In some experiments after 5th washing, 6 ml of 1/6 anti-Sindbis rabbit serum in BAF medium was added for 30 minutes. After the last washing, 10 ml of fresh BAF medium was added to the culture. Cultures were incubated in stationary position. At intervals, 0.2 ml of fluid was removed, diluted 10-fold in BAF medium and stored at –20°C. Fluids from one experiment were assayed simultaneously by plaquing on chick embryo cultures. Four experiments employing cultures prepared from different lots of cells gave results consistent in each case with those depicted in Fig. 5 for 2 experiments. The interval preceding rapid increase in extracellular virus was consistently longer in young cultures. Subsequently concentration of virus was consistently 2 to 3 $\log_{10}$ units lower in young cultures. These differences were outside the limits of error of assay. Nor did they depend upon differences in number of cells. The range of cell counts from 7 cultures varying 5 to 30 days in age was 8-37 $\times 10^5$. To account for observed discrepancies in titer, a difference of the order of 100-1000 × in cell population would be required. In these and comparable experiments, cultures of 30 days or older consistently degenerated completely within 5 days after addition of virus, whereas in about one-half of young cultures tested, no signs of degeneration were observed. In the remainder, the cells were completely destroyed. Fig. 6 depicts virus titers in fluid phase of 4 cultures which showed no degeneration. Up to 15 days after inoculation the titers are similar. Excluding the bottle which was reinoculated with Sindbis virus, measurements made after 15 days showed no consistent pattern. In spite of the presence of large quantities of virus in cultures over 30 days of age no degeneration was noted comparable to that which occurred regularly in cultures of this age inoculated for the first time. Variations in viral titer could not be correlated with changes of fluid. In all cultures cells continued to metabolize throughout experiment.

It seemed possible that differences in viral concentrations observed in young and old cultures might depend on retention of a greater proportion of virus within young cells. To explore this possibility a small amount of fluid was removed after 7 hours incubation from an old and a young culture treated identically with those in one of the experiments depicted in Fig. 5. Cells with remaining fluid were then ground with sand and centrifuged. Aliquots of supernatant fluids together with specimens previously collected were assayed for viral activity. The titers of these supernatant fluids were comparable to those of aliquots first removed, which were of the same order as shown in Fig. 5. These findings indicate that virus in significant quantities had not been generated and retained within the young cells.

Discussion. In certain cultural systems rolling does not appear to affect final cytopathogenic effect of poliovirus(12,13), although it may accelerate it(14). Our results show that with at least one strain of poliovirus, rolling under certain conditions may greatly influence expression of viral cytopathogenicity. Less attention has been paid to effects of aging of primary cell cultures. It has been demonstrated that synthesis of herpes simplex virus does not occur in agar slant cultures of chick embryo fragments that are older than 4 days(15). The opposite effect of aging was recently reported by Kumagi and co-workers(16) who found that in swine testicle cell cultures of less than 5 days, synthesis of Newcastle disease virus was reduced and cytopathic changes were absent. In 6-8 day cultures viral multiplication was increased and cell destruction became widespread. The results here described afford additional examples of resistance of young cultures and indicate that the period of such resistance may extend for as long as 2-3 weeks. The factors underlying this early resistance in primary cultures of human amnion cells remain obscure. Our analysis, as well as results of a preliminary experiment, suggest that most cells in young cultures do not synthesize virus. If this hypothesis proves correct, it will be necessary to discover whether the refractory state depends upon failure of virus to attach to and enter these cells or upon its subsequent failure to multiply.

A further objective consists in determination of whether resistance is conditioned by unnatural environment of culture or whether it reflects the temporary retention *in vitro* of a property normally present *in vivo*. It will be recalled that poliovirus can be latent in the kidney of living monkeys without causing manifest injury. When cultured, however, cells from the infected kidney are subsequently destroyed(17).

From a practical point of view, these results indicate that in the study of viral agents whose behavior in tissue culture is uncertain, disregard of the effects of cultural age and agitation may be misleading.

Summary. 1) The cytopathic response of primary human amnion cell cultures to chick embryo adapted Type II poliovirus was absent in stationary cultures inoculated at 5-10 days of age. In stationary cultures inoculated when 30 or more days of age, complete destruction of cells followed. Multiplication of virus in young cultures occurred in the absence of recognizable cytopathic change. Cytopathic response of cultures of approximately 14 days was markedly enhanced by rolling after inoculation. The effect of rolling was not clearly apparent when other strains of poliovirus including attenuated strains were tested. 2) Frequently the cytopathic response of amnion cultures to Sindbis virus was reduced or absent in young as compared with old cultures, but response of young cultures was variable. No effect of rolling on cytopathic response could be demonstrated. Viral multiplication was greater in older cultures.

I am grateful to Dr. J. F. Enders for guidance.

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A Micrometer Plunger Adaptation to the Stern-Kirk Microrespirometer.
(24678)

REIDAR WENNESLAND, ALVIN D. SENTER AND JOHN J. EILER
University of California Schools of Medicine, Department of Medicine, Dentistry, and Pharmacy, San Francisco

The Stern-Kirk microrespirometer(1) has a number of features which make it well suited for studies of respiration in small samples of tissue. It is sturdy, easy to operate and sensitive. The respiration chamber and thermobarometer compensation chamber consist of 2 recesses drilled in a metal block base. The metal quickly equilibrates temperature differences between the 2 chambers, so that accuracy of the apparatus is not affected by small changes in surrounding temperature. Since the chambers are closed during experiments, repeated thermobarometer corrections are unnecessary. In this respect, the Stern-Kirk respirometer resembles volumetric respirometers of similar construction(2-5). Unlike them, however, it requires time-consuming calibration procedures. To overcome this

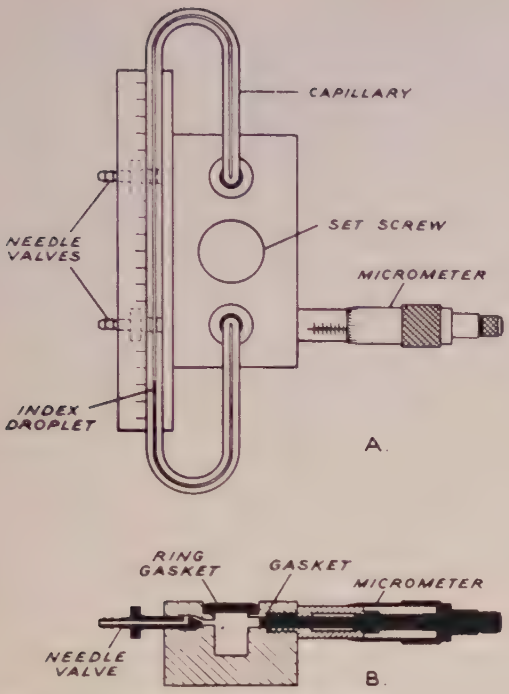

FIG. 1. Stern-Kirk microrespirometer with micrometer plunger adaptation. A. Top view, completely assembled. B. Cross-section of lower part with plunger attached.

drawback, the respiration chamber was provided with a micrometer plunger which gives the apparatus the ease of calibration and operation of certain volumetric respirometers(3-6), without sacrificing its own advantageous features.

Methods. A stainless steel plunger is inserted into the respiration chamber through an opening drilled in the metal block base. The entry is made airtight with a gasket. Any alteration in volume of gases in the respiration chamber is reflected by change in position of the index droplet in the capillary which connects respiration chamber with compensation chamber. At intervals the plunger is moved until the droplet has been returned to its original position at beginning of experiment. Readings of alterations in gas volume are made by determining the change in plunger's position. This can be done accurately with a variety of devices, such as a micrometer scale, revolution counter(6) or dial micrometer gauge(7). Measurement of the plunger's diameter with a micrometer caliper, is the only calibration required. The sensitivity and range of instrument can be extended by using interchangeable plungers of various diameters in combination with capillaries of different dimensions. The apparatus is shown in Fig. 1; a commercal micrometer caliper head provides the plunger and reading device. The plunger has a diameter of ⅛ inch. The micrometer permits ½-inch longitudinal displacement divided into 500 scale units. This corresponds to total volume displacement of 100.4 mm^3 or 0.201 mm^3/scale unit. In Fig. 1A respirometer and micrometer head is seen from above. In Fig. 1B a cross section of bottom part of instrument shows point of entry of plunger into the respiration chamber opposite the hole of the needle valve. The latter allows gassing of chambers and prevents loss of index droplet during opening and closing of respirometer (1). The gasket around plunger is made of Teflon.

Suspending medium, alkali and tissue are introduced as usual, and respiration chamber is oxygenated. The respirometer cover is secured with the set screw, and needle valves are closed. Location of the index droplet in the capillary is noted after temperature equilibration. Position of the plunger is then read on the micrometer scale. Starting point of the droplet can be adjusted by tilting instrument prior to closing the needle valves(1). In studies of oxygen consumption, droplet will slowly advance toward respiration chamber. At regular intervals it is returned to its starting point by moving plunger deeper into the chamber. The specimen can be shaken, if re-

TABLE I. Cumulative Oxygen Uptake in 60 Min. by 7.28 mg Wet Weight of Human Gingival Tissue Using the Stern-Kirk Microrespirometer with the Micrometer Plunger Adaptation.

Time (min.)	Position of plunger on micrometer scale	Plunger displacement in micrometer units	mm^3 O_2 consumed (displ. × 0.201)
0	387	0	0
9	379.5	7.5	1.5
17	373.5	13.5	2.7
25	368	19	3.8
30	365	22	4.4
40	360	27	5.4
60	351.5	35.5	7.1

The Qo_2 (wet wt) = 0.97; Qo_2 (nitrogen) = 31.8 38°C.

quired, by placing the respirometer on any commercial shaker or vibrator with adequate speed of motion.

Example of use. The adapted instrument was used to measure oxygen uptake of human gingiva obtained by surgical excision(8). Position of micrometer plunger at various time intervals and cumulative oxygen uptake at 38°C are shown in Table I. Wet weight of the respiring sample was 7.28 mg or 0.223 mg nitrogen as determined by Kjeldahl nesslerization.

Summary. A Stern-Kirk microrespirometer was provided with a plunger adaptation which obviates the need of prior calibration of volumes of respiration and compensation chambers. By combining interchangeable plungers of different diameters with various available capillary manometer tubes, the range of the instrument can be extended. Use of the instrument in measuring the oxygen uptake of human gingival tissue is described.

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Reversal of Inhibitory Activity of Chlorpromazine by Calcium.* (24679)

H. VASKEN APOSHIAN, NANCY S. POINTER AND MARY M. APOSHIAN
(Introduced by A. D. Bass)
Dept. of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tenn.

Since the use of microorganisms has been a valuable aid in discovery of new and the elucidation of known metabolic reactions, a program has been initiated in which bacteria were used to obtain information as to possible mode of action of various drugs affecting the central nervous system. The present report concerns inhibition of growth of *E. coli* by chlorpromazine, a "tranquilizer," and its reversal by calcium.

Methods. *E. coli* A.T.C.C. #9723 was maintained and the inoculum prepared essentially by a previously described method(1). All assays were performed at 37° for 17 hours in final volume of 10 ml, using Dunn's(1) modification of Anderson's salts, ammonium chloride and glucose medium(2).

Results. As shown in Fig. 1, 0.075 μmole of chlorpromazine/ml of medium produces 50% inhibition of maximum growth of this organism, whereas 0.14 μmole of promazine, a less active "tranquilizer" for humans, is necessary for 50% inhibition of growth. Since these 2 drugs also possess antihistaminic activity, tripelennamine (a commonly used antihistaminic) was tested and found devoid of significant inhibitory activity under our conditions.

Various compounds, either metabolites similar in structure, neurohumoral agents or substituents of coenzymes, when used in concentrations that alone were not inhibitory for this strain of *E. coli*, did not reverse inhibitory activity of chlorpromazine or promazine. Examples of such compounds are histamine, histidine, acetylcholine, epinephrine, norepinephrine, 5-hydroxy-tryptamine, nicotinic acid, nicotinamide, thiamine, riboflavin, biotin, pyridoxine, pyridoxal phosphate, or pyridoxamine.

Calcium-d-pantothenate, however, reversed the inhibitory activity of chlorpromazine. Since the amount of pantothenate required for reversal was greater than that expected to be

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necessary for catalytic purposes, it was thought that reversal of the inhibitory activity of chlorpromazine might be due to the calcium ion rather than the pantothenate moiety. The reversing activity of calcium-d-pantothenate was compared with that of calcium chloride or calcium acetate. Table I shows that calcium in the form of calcium-d-pantothenate, calcium chloride, or calcium acetate reverses the inhibitory activity of chlorpromazine. Sodium chloride or sodium acetate is unable to reverse inhibitory activity of chlorpromazine.

It is of interest to note that chlorpromazine is twice as active as promazine as an inhibitor of this organism and that the former is twice as potent as the latter as an ataractic in humans. Woolley has recently shown that the muscle contracting activity of 5-hydroxytryptamine is associated with a change in permeability of the muscle cell to calcium ions (3). It would be of interest to investigate the relationship between chlorpromazine and permeability of mammalian cell membranes to calcium to elucidate the mode of action of this "tranquilizer" in humans.

Summary. Chlorpromazine inhibits growth of *E. coli* 9723. Various compounds, either metabolites similar in structure, neurohumoral agents or substituents of coenzymes, do not reverse inhibitory activity of this "tranquilizer." Calcium in the form of calcium-d-pantothenate, calcium chloride or calcium acetate reverses the inhibitory activity of chlorpromazine.

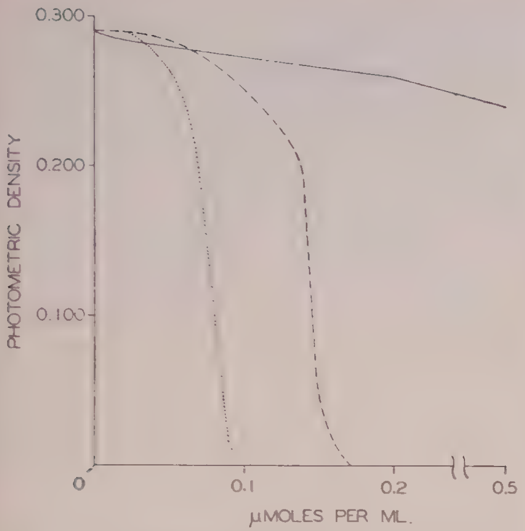

FIG. 1. Influence of chlorpromazine, promazine or tripelennamine on 17-hr growth of *E. coli* #9723. Chlorpromazine (•••••), promazine (– – – –), tripelennamine (———).

TABLE I. Reversal of Inhibitory Activity of Chlorpromazine by Calcium-d-pantothenate, Calcium Chloride or Calcium Acetate.

		Supplements		
Chlorpromazine	Amt of supplement	Calcium-d-pantothenate	Ca. chloride	Ca. acetate
μmoles/ml		Photometric density*		
.0	.0	.299	.299	.299
.1	.0	.008	.008	.008
.1	.1	.030	.040	.038
.1	.2	.174	.156	.192
.1	.3	.200	.204	.215
.1	.4	.234	.238	.244
.1	.5	.265	.270	.267
.1	.6	.281	.273	.272
.0	.1	.310	.301	.301
.0	.2	.299	.303	.310
.0	.3	.308	.304	.304
.0	.4	.309	.305	.308
.0	.5	.306	.310	.311
.0	.6	.306	.303	.303

Test organism, *E. coli* #9723, incubated for 17 hr at 37°.

* A measure of culture turbidity. Photometric density = 2 − log G where G equals galvanometer reading of Evelyn Photoelectric Colorimeter.

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Submicroscopic Morphology of Avian Neoplasms. III. Studies on Visceral Lymphomatosis.* (24680)

L. Dmochowski, C. E. Grey, B. R. Burmester and M. A. Gross
Section of Virology and Electron Microscopy, University of Texas Anderson Hospital and Tumor Inst.; Dept. of Microbiology, Baylor University College of Medicine, Houston; and U. S. Dept. of Agric., Regional Poultry Research Lab., East Lansing, Mich.

The natural occurrence of visceral lymphomatosis (lymphoid leukosis lymphocytoma), one of the diseases of fowl leukosis, is widespread, the disease being transmitted from parent to offspring *via* the egg and also by contact with infected environment(1). Experimental transmission may be effected by infective material, such as plasma, oral washings, and various extracts (tumors, embryonated eggs, feces) from either naturally infected chickens or those used in serial passage of the virus. In most cases, exposure of susceptible chickens to all the different sources of virus, has resulted in development not only of visceral lymphomatosis but also of erythroblastosis. Relative and absolute occurrence of each disease is subject to marked variation and appears influenced by effective dose of virus and genetic variation of host(2,3). It remains to be determined whether these 2 disease entities are caused by specific viruses or by a single multipotent virus. The present studies were undertaken to characterize any submicroscopic changes and specific structures which may be found in organs of chickens with visceral lymphomatosis acquired after natural and experimental exposure.

Materials and methods. Chickens with visceral lymphomatosis induced experimentally with RPL-12 lymphoid tumor, and others with the disease acquired under natural conditions were used. The RPL-12 tumor strain originated as a field case(4). Cell-free preparations of this tumor fail to induce tumors at site of inoculation but produce a high incidence of lymphomatosis tumors of viscera in chickens(5). Visceral lymphomatosis occurs also in chickens in contact with other birds inoculated with filtrates of strain RPL-12 tumor. They induce, after 4 months, visceral lymphomatosis characterized by extravascular proliferation of lymphoid cells in one or more viscera(2,3). A secondary feature in some chickens is the intravascular involvement with a character of erythroblastic leukosis(2). Donor birds had visceral lymphomatosis induced by intravenous inoculation of chickens at age 1 day to 3 weeks, with cell free filtrates of different passages of strain RPL-12 tumor. Chickens which contracted the disease naturally, or by contact with inoculated birds were also used as donors. Spleen from chickens showing moderate or extensive tumor deposits, histologically diagnosed as visceral lymphomatosis was studied in ultrathin sections by means of RCA EMU-3A electron microscope. The method of tissue preparation for study in ultrathin sections was the same as that used for erythroblastosis(6) and granuloblastosis(7).

Results. The cytoplasm of tumorous cells of spleen from chickens with visceral lymphomatosis showed alterations in mitochondria, endoplasmic reticulum, and cellular membranes, while the nucleus usually remained unchanged. Osmiophilic bodies of varying size and appearance were also frequently encountered. A general view of appearance of sections of the affected spleen from chickens with experimentally induced visceral lymphomatosis is shown in Fig. 1. As can be seen, not all tumor cells show changes. The appearance of several cells at somewhat higher magnification may be seen in Fig. 2. Numerous osmiophilic bodies of varying size and different appearance are present. In addition, structures resembling virus particles may be seen within the osmiophilic bodies and scattered in the cytoplasm (Fig. 2). A cell with only a few inclusion-like bodies is shown in Fig. 3. The cell also contains a number of different size cytoplasmic vacuoles filled with

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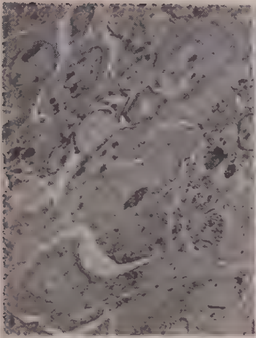
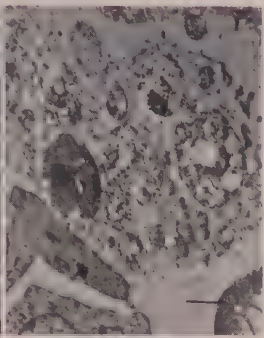
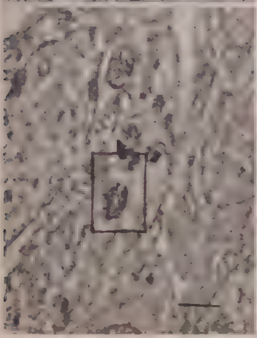






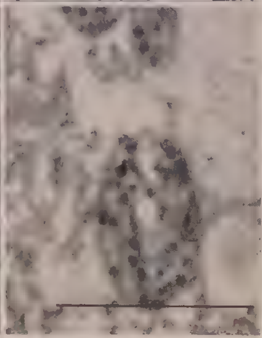


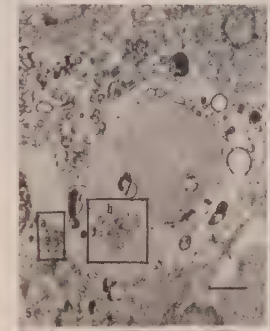
FIG. 1. Electron micrograph of section of tumorous cells of spleen from chicken with visceral lymphomatosis. Dense osmiophilic bodies present in cytoplasm of some cells. Magnification 1,700 ×.

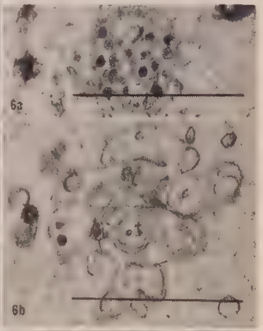
FIG. 2. Cytoplasm of tumorous cells of spleen with inclusion-like bodies of different size and appearance and characteristic particles. Magnification 4,750 ×.

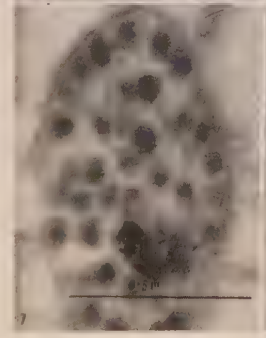
FIG. 3. Inclusion-like bodies with characteristic particles and vacuoles present in the cytoplasm of cell surrounded by apparently unaffected cells. Magnification 4,750 ×.

FIG. 4. Inclusion-like body from Fig. 3 with particles showing characteristic internal structure. Magnification 24,000 ×.

homogeneous material, and is surrounded by cells showing no apparent alterations. An inclusion-like body, similar to one shown in Fig. 3, may be seen at higher magnification in Fig. 4. It is in part surrounded by what appears to be a membrane, and contains a number of characteristic particles, showing a dense central core with an outer lighter zone, surrounded by external limiting membrane. Appearance of a disintegrating cell or cells is shown in Fig. 5. An inclusion-like body, marked by letter "a", may be seen at higher magnification in Fig. 6a. Characteristic particles, resembling virus particles, are surrounded by what appears to be a limiting membrane. Part of Fig. 5, marked by letter "b", may be seen at higher magnification in Fig. 6b, which shows characteristic membranous structures, frequently encountered in cytoplasm of affected cells. The changes described and characteristic particles were found in approximately one out of 50 tumor cells examined. The characteristic appearance of particles within inclusion-like bodies and in intercellular spaces may be seen in Fig. 7 and Fig. 8. The particles, whether within inclusion-like bodies or outside the cells, are of similar appearance. An estimation of the size of 700 particles, measured both within cells and in intercellular spaces, showed average size of smallest particles to be approximately 640 Å and that of largest particles approximately 820 Å. Overall average size of particles was 720 Å and average size of internal dense core approximately 300 Å.







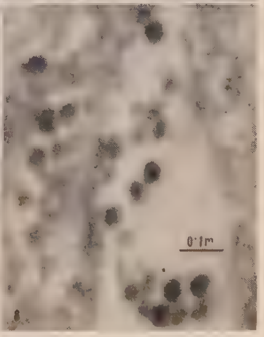


FIG. 5. Appearance of breakdown cell or cells, surrounded by comparatively unchanged cells. Magnification 4,250 ×.

FIG. 6a and 6b. Parts of Fig. 5 enlarged. Characteristic structure of particles within inclusion-like body. Membranous structures probably originating from endoplasmic reticulum. Magnification 21,250 ×.

FIG. 7. Appearance of characteristic particles within an inclusion-like body surrounded by a membrane. Magnification 56,250 ×.

FIG. 8. Appearance of similar particles in an intercellular space. Magnification 45,000 ×.

Discussion. Study of ultrathin sections of tumorous spleens from chickens with naturally acquired and experimentally induced visceral lymphomatosis showed a number of similar characteristic changes in cytoplasm of some tumor cells. Inclusion-like bodies, some with spherical or spheroidal structures resembling virus particles were frequently observed. The particles were also found in cytoplasmic vacuoles, occasionally in cytoplasm and frequently in intercellular spaces. Other changes in cytoplasm of tumor cells, such as vacuolization, breakdown of mitochondria, swelling and breakdown of endoplasmic reticulum, and of cellular membranes, although not strictly specific for viral infections, have usually been observed in association with the presence of inclusion-like bodies and characteristic viral structures. Close examination of the inclusion-like bodies revealed the surrounding membrane to be composed in places of 2 membranes and presence of what could be interpreted as parts of broken-down cristae. It appears that some inclusion-like bodies may be of mitochondrial origin. Similar bodies were described in erythroblastosis and their origin attributed to mitochondria(8). Characteristic "whorl-like" formations of membranous structures have frequently been observed in some tumor cells of the affected spleen. Their origin appears to be from endoplasmic reticulum and they may be an expression of cellular defense mechanism. Somewhat similar membranes have been observed in other virus-cell systems, such as Ehrlich ascites tumor cells infected with Newcastle Disease Virus(9) and in monkey kidney cells infected with mumps virus(10).

Similar changes and characteristic particles have been observed in erythroblastosis(6) and in granuloblastosis(7). The particles observed in these 2 diseases, although similar in structure were consistently somewhat smaller in size than those encountered in visceral lymphomatosis.

Spleen of young control chickens failed to reveal similar particles, although they have been observed by others in approximately 10% of control birds(8).

Summary. Ultrathin sections of tumorous spleen from chickens with visceral lymphomatosis acquired after natural and experimental exposure have been examined in the electron microscope. Spherical and spheroid particles were observed within inclusion-like bodies present in cytoplasm of cells, in cytoplasmic vacuoles, and in intercellular spaces. The particles have an internal dense zone of about 300 Å, surrounded by a less dense zone and limited by an outer membrane of a size varying from 640 Å to 820 Å, and average diameter of 720 Å. These particles resemble in appearance but not in size particles observed in erythroblastosis and granuloblastosis of chickens.

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Submicroscopic Morphology of Avian Neoplasms. IV. Studies on Erythroblastosis of Strain RPL-12.* (24681)

L. Dmochowski, C. E. Grey and B. R. Burmester
Section of Virology and Electron Microscopy, University of Texas Anderson Hospital and Tumor Inst.; Dept. of Microbiology, Baylor University College of Medicine, Houston; and U. S. Dept. of Agric., Regional Poultry Research Lab., East Lansing, Mich.

Studies have recently been reported on submicroscopic morphology of avian erythroblastosis(1) caused by strain "R" of Engelbreth-Holm(2) and extensively investigated by Beard(3). Erythroblastosis is also caused by strain "RPL-12," which originally caused primarily visceral lymphomatosis, but after serial passages elicited erythroblastosis when given in large doses to susceptible chickens. Both strains in low doses also induce a high incidence of visceral lymphomatosis(4,5). The most notable biological differences between these 2 strains are range of time of death (12-30 days for strain R, 30-90 days for strain RPL-12) and occurrence of osteopetrosis marked with strain RPL-12 and negligible with strain R. Majority of chickens, when inoculated to age of 84 days with cell-free preparations of RPL-12 strain, die of erythroblastosis(4), while older chickens when inoculated with similar preparations, develop more frequently visceral lymphomatosis but only after 100 days, irrespective of age of inoculation. It has been suggested that visceral lymphomatosis virus is a "wild type" virus, since in contrast to erythroblastosis virus it has maintained itself in most poultry populations for several decades(6). The present studies were carried out as a first step in ascertaining relationship of agent or agents causing visceral lymphomatosis and erythroblastosis RPL-12 strain.

Material and methods. Chickens which developed erythroblastosis following intravenous or intraperitoneal inoculation with cell-free preparations of RPL-12 tumor strain, served as donors. Spleen from chickens with macroscopic and microscopic changes diagnostic of erythroblastosis was used for ultrathin section studies in an RCA EMU-3A electron microscope. Method of specimen preparation for electron microscopy was the same as in study of organs from chickens with erythroblastosis caused by "R" strain of Engelbreth-Holm(1), granuloblastosis(7), and visceral lymphomatosis(8).

Results. The general appearance of some tumor cells from spleen of chickens with erythroblastosis RPL-12 strain, as frequently encountered during scanning, may be seen in Fig. 1. Cytoplasm and nucleus of cells appear unchanged. Nuclear and cytoplasmic membranes may be seen clearly delineated and intercellular spaces are well defined. Frequently, however, (Fig. 2), at somewhat higher magnification dense spherical particles have been encountered in clusters or in rows within intercellular spaces and in what appear to be vacuoles in the cytoplasm of some cells. Majority of cells appear unchanged, although some vacuoles may be seen in the cytoplasm of a cell in lower left hand corner. A cluster of particles within what may be a vacuole in the cytoplasm of a cell in Fig. 2, may be seen at higher magnification in Fig. 3. Some particles show a characteristic internal structure, with a central dense core and a limiting membrane surrounding a less dense zone around the central core. Similar spherical or spheroid particles have been observed scattered within the cytoplasm of cells and in inclusion-like bodies, (Fig. 4). Appearance of some tumor cells, frequently observed, may be seen in Fig. 5. Numerous dense osmiophilic bodies of varying size and appearance are a striking feature. Cell membranes have disappeared and cytoplasm of cells contains numerous altered mitochondria and vacuoles, some of considerable dimensions, partly filled with irregularly shaped dense osmiophilic material. In addition, inclusion-like bodies may be seen containing spherical particles. Some particles

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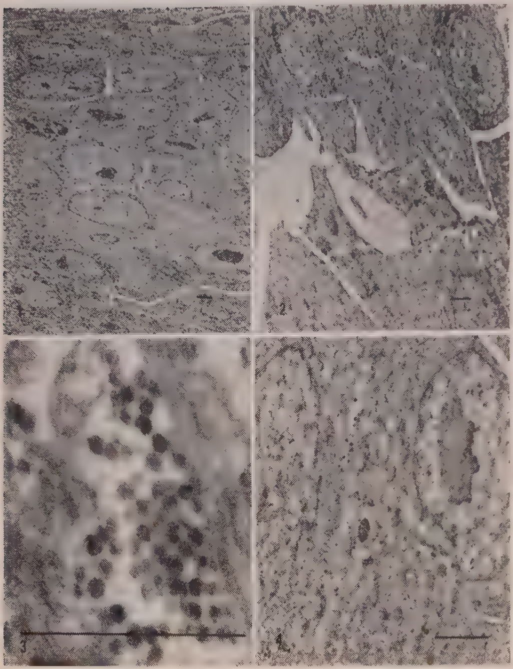

FIG. 1. General view of an ultrathin section of tumorous spleen from chicken with erythroblastosis RPL-12 strain. Magnification 1,700 ×.

FIG. 2. Characteristic particles present among apparently unchanged cells of tumorous spleen. Magnification 2,500 ×.

FIG. 3. Part of Fig. 2 showing characteristic particles. Magnification 28,000 ×.

FIG. 4. Dense inclusion-like body with particles, also present in intercellular spaces. Magnification 6,000 ×.

are also scattered in the cytoplasm and in spaces between broken down cell material. Part of cytoplasm of a tumor cell, with an inclusion-like body containing characteristic particles, some lying free in the cytoplasm, is shown in Fig. 6. Characteristic membranous structures occasionally observed in breakdown cells may also be seen. Comparison of appearance of characteristic particles within an inclusion-like body and those scattered within and outside the cytoplasm of a cell is shown in Fig. 7 and Fig. 8. Particles shown in both Figures reveal the same characteristic structure. Those within the inclusion-like body appear more uniform in size than those outside the body.

Calculation of size of 700 particles present within inclusion-like bodies and outside the cells, showed average size of smallest to be approximately 650 Å and that of largest particles approximately 830 Å. The overall average size of all particles has been estimated at 730 Å. The size of their dense core ranged from 280 Å to 380 Å, average 320 Å.

Discussion. Electron microscope studies of spleen affected by visceral lymphomatosis(8) and erythroblastosis RPL-12 strain, revealed an essential similarity in changes of tumor cells in these 2 conditions, and also a similarity in size and frequency of appearance of characteristic virus-like particles present in affected organs. Average size of particles, based on estimation of 700 particles in each of the 2 diseases was similar, 720 Å and 730 Å respectively. The morphological similarity of particles, assuming they are the causative agent, could conceivably indicate that the same agent

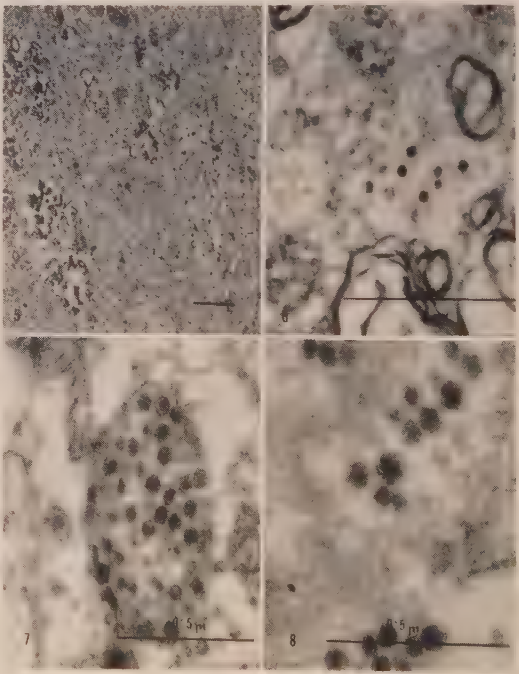

FIG. 5. General view of an ultrathin section of tumorous spleen from chicken with erythroblastosis RPL-12 strain, showing breakdown cells with dense osmiophilic bodies and particles. Magnification 4,800 ×.

FIG. 6. Part of a cytoplasm of breakdown cells with characteristic particles within an inclusion-like body and in the cytoplasm. Whorl-like formations of membranous structures. Magnification 21,000 ×.

FIG. 7. An inclusion-like body with characteristic particles. Magnification 37,000 ×.

FIG. 8. Characteristic particles lying free in and outside the cytoplasm of an affected cell. Magnification 45,000 ×.

is involved. Frequent induction of visceral lymphomatosis and erythroblastosis RPL-12 strain by the same cell-free preparations from naturally occurring cases of visceral lymphomatosis may indicate variation or mutation of the same agent(4). Further studies, however, are required to settle the problem.

Changes observed in organs of chickens with erythroblastosis "R" strain(1) and granuloblastosis(7) are essentially similar to those seen in visceral lymphomatosis(8) and erythroblastosis RPL-12 strain. However, the average size of the characteristic particles, based on a count of approximately 700 particles, is 640 Å in erythroblastosis "R" strain and 620 Å in granuloblastosis. A similarity in size and structure of particles present in granuloblastosis(7) and erythroblastosis "R" strain(1), however, does not necessarily indicate their identity, in view of observed chemical and serological differences between the agents of these 2 diseases(9).

The average size of particles in visceral lymphomatosis and erythroblastosis RPL-12 strain, based on the average of 700 measurements, would appear to be larger than the average size of particles in erythroblastosis "R" strain and granuloblastosis, but further statistical analysis will have to be made before unequivocal conclusions can be drawn about the observed difference in size of the particles.

It has been suggested, although evidence is far from conclusive, that all forms of avian leukosis and related neoplasms are caused by a single multipotent virus(10,11,12). Evidence accumulated during recent years indicates, however, that viruses of avian leukemias, erythroblastosis, and myeloblastosis (3), virus of lymphomatosis(13), and viruses responsible for numerous avian sarcomas, although related agents, are in fact separate and distinct biological entities(3). The simultaneous application of as many technics as possible to the study of these viruses, such as biological, biophysical, biochemical as well as others and also of electron microscopy of ultrathin sections of tissues and organs infected by these viruses, may possibly settle the problem of the relationship of these agents.

Summary. Tumorous spleen from chickens with erythroblastosis RPL-12 strain were examined in ultrathin sections. The cytoplasm of cells of the affected spleens showed changes similar to those observed in visceral lymphomatosis. Characteristic particles were observed in cytoplasm of cells similar in structure and size to that of particles in visceral lymphomatosis. The diameter of the particles was found to vary from 650 Å to 830 Å, with an average of 730 Å, and that of the internal dense core varied from 280 Å to 380 Å, with an average of 320 Å. The average size of the characteristic particles in erythroblastosis RPL-12 strain and visceral lymphomatosis is similar and appears to be larger than that of similar particles in erythroblastosis "R" strain and granuloblastosis (myeloblastosis).

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Changes in Body Weight of Normal and Castrated Rats by Different Doses of Testosterone Propionate.*† (24682)

CHARLES D. KOCHAKIAN AND BARBARA R. ENDAHL
Dept. of Physiology, University of Alabama Medical Center, Birmingham

Ability of testosterone propionate to increase body weight of castrated rats fed constant amounts(1) or *ad lib.*(2) is well established. The normal adult rat when fed constant food intake also responds to testosterone propionate by increase in body weight(3) but when fed *ad lib.* shows an entirely different response. It loses body weight(4). In view of this difference in response between normal and castrated rats fed *ad lib.*, a study of the effect of various doses of testosterone propionate was undertaken.

Procedure. Male rats (Holtzman) were castrated at 110 g body weight (40 days old) and another similar age group kept as normals. Rats were kept in individual cages in air-conditioned room at 25.5-26.5°C. Their diet was: casein 900, sucrose (granulated sugar) 300, celluflour 100, yeast (Fleischmann's No. 2019) 500, hydrogenated vegetable oil (primex) 400, Wesson's salt mixture 200, cod liver oil (Patch's) 12, and 30% tocopherol mixture.‡ Amount of food eaten and body weights of rats were determined 3 times/week. Daily subcutaneous injections of testosterone propionate‡ were begun 35 days after castration. Androgen was diluted with sesame oil so that each daily dose was contained in 0.05 ml of oil. Normal and castrated control animals received injections of sesame oil.

Results. Two sets of experiments were performed. In the first (Fig. 1A, 2A), 0.025 to 0.1 mg/day of testosterone propionate were used and in the second (Fig. 1B, 2B), 0.1 to 2.5 mg/day. Number of rats in each experiment is indicated in footnotes of Fig. 1, 2.

Normal rats. Testosterone propionate initially produced a slightly greater gain in body weight (Fig. 1A, B) maintained at only lowest dose (0.025 mg/day). Other doses produced a decrease in rate of body weight gain. Onset and degree of decrease was related to dose. At end of experiment every group of androgen treated rats except those at lowest dose had body weights less than those of control rats. Maximum decrease was attained at dose of 0.5 mg/day (Fig. 3). Further increase in dose did not produce any greater loss in body weight. On cessation of injections, loss in body weight continued for about another week, then an increase occurred, most marked in animals that received the higher doses of androgen (Fig. 1B). Food intake was increased above that of controls, in rats at low dose levels (Fig. 1A) while that in high dose levels (Fig. 1B), was decreased for about 3 weeks, then gradually returned towards that of controls. On cessation of injections a noticeable increase in food intake occurred in rats which received more than 0.1 mg/day. Thus, food consumption of rats in the respective groups varied with changes in body weights.

Castrated rats. Testosterone propionate produced an increase in rate of body weight gain of castrated rats at every dose level (Fig. 2A, B). Maximum increase in body weight, however, became less at doses above 0.1 mg/day (Fig. 3). Continuation of androgen treatment, moreover, resulted in a decrease in rate of body weight gain in those animals receiving more than 0.1 mg/day. Onset and degree of decrease was related to dose of androgen (Fig. 2A, B, 3). The extent of change was such that rats receiving 0.5 and 1 mg/day lost the equivalent of practically all the stimulated increase in weight and rats at 2.5 mg/day had lost more than androgen-induced increase in body weight.

All groups except those at the 2 highest dose levels showed initially an increase in food intake which later changed in parallel with

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† John Hill and Rayburn Key provided valuable technical assistance.

‡ Tocopherol concentrate was generously provided by Distillation Products Ind.

§ Testosterone propionate was kindly provided as perandren by Ciba Pharmaceutical Products.

maximum increases in body weight. After about 10 days food intakes returned toward control level, then increased again (Fig. 2A, B). Cessation of injections was followed within a few days by increase in food intake. This effect was most evident at higher dose levels.

Discussion. Testosterone propionate can inhibit body weight gain of both normal and castrated young adult rats. Inhibition, however, is preceded by an initial stimulation, slight and brief in normal rats, very marked in castrated rats. In both instances degree of stimulation is most evident at very low doses of androgen and becomes less at high dose levels.

The difference between normal and castrated rats seems to be due to metabolic status of the animals. When the castrated rat has been restored to normal state by exogenous androgen, it then shows the same or similar type of response in body weight and food intake as the normal rat. Thus, administration of excess androgen results in loss of body weight probably due to greater utilization of body fat in compensation for a decrease in food intake(*cf.* 4) and only a very slight inhibition of body growth(2,4). Thus, the changes represent not only protein-anabolic(1,2) but also fat-catabolic(2,4) action of the androgen.

The course of changes in body weight of castrated rats is very similar, indeed practically identical, to that of androgen-treated rats on a constant food intake(1). In both instances androgen produces an initial increase in body weight and with continued injection, a loss in body weight. Furthermore, this loss is accompanied by loss in body fat(2,4). The greater gain in body weight by rats fed *ad lib.* very likely is due to simultaneous increase in food intake. Thus, new tissue is formed not only through protein-anabolic action of the androgen but also to ingestion of extra foodstuffs. The initial gain in body weight of an-

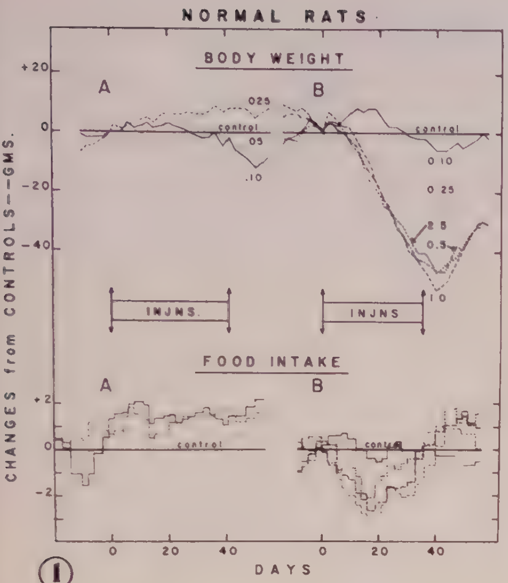

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FIG. 1. Changes in body wt and food intake in normal rats inj. with various doses of testosterone propionate. Changes are from control rats which during inj. period gained 107 g in series A and 90 g in series B. Avg body wt at beginning of inj. were: Series A 301-315 g and series B 276-283 g per group. Series A contained 10 rats/group and series B, 5 to 8 rats/group. Dose of testosterone propionate (mg/day) is indicated beside body wt curves of respective groups.

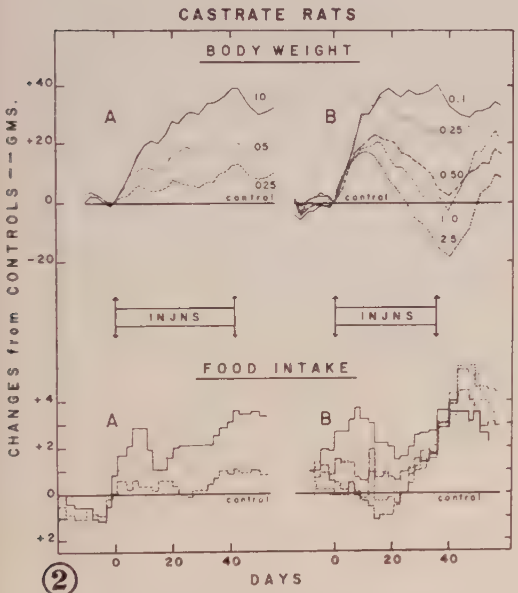

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FIG. 2. Changes in body wt and food intake in castrated rats inj. with various doses of testosterone propionate. Changes are from control rats which during inj. period gained 107 g in series A and 77 in series B. Avg body wt at beginning of inj. were: Series A 254-260 g/group and series B 274-294 g/group. Series A contained 10 rats/group and series B 5 to 7 rats/group. Dose of testosterone propionate (mg/day) is indicated beside body wt curves of respective groups.

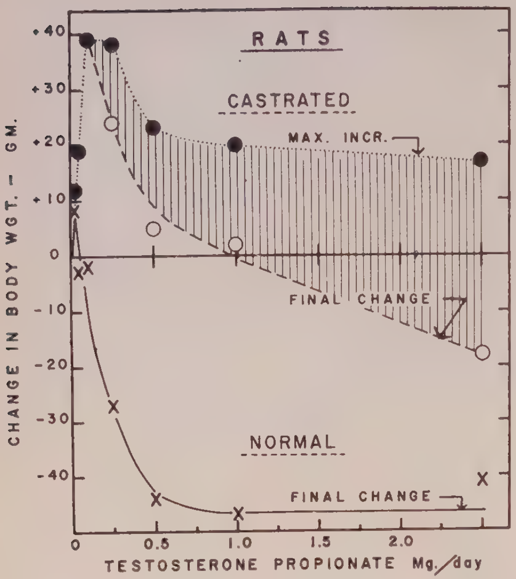

FIG. 3. Comparison of effect of dose of testosterone propionate on body wt of normal and castrated rats. See Fig. 1 and 2 for details.

drogen-treated normal rat fed *ad lib.* is much less than that of the normal rat on a constant food intake(3), and loss in body weight on continued injection is much greater.

Depression of normal body weight gain by testosterone propionate at doses above 0.1 mg/day in the growing castrated rat indicates that the physiological need of the rat is well below this level. Considerable stimulation was observed at the very low dose of 25 μg/day. Body weight gain of immature normal rats also has been reported to be stimulated by small doses(5,6,7) and depressed by large doses(6,8,9)‖ of androgen. Other reports, however, indicated no effect(9,10,11) especially when low doses of androgen were administered.

Summary. Administration of testosterone propionate above physiological levels inhibits body weight gain of normal and castrated young adult rats. Inhibition in the normal rat appears in doses above 25 μg/day and in castrated rats at above 100 μg/day. In the castrated rat inhibition is preceded by increase in rate of body weight gain. Food consumption of both normal and castrated rats varied with changes in body weight.

‖ Large doses of testosterone propionate produce remarkable inhibition of growth in chicks (12) but have no effect on male guinea pig(13). Other species show responses similar to those in the rat.

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Direct Stimulation of Adrenocortical Secretion by Synthetic Vasopressin in Dogs.* (24683)

JAMES G. HILTON, LOUIS F. SCIAN,† COENRAAD D. WESTERMANN
AND OSCAR R. KRUESI (Introduced by Joseph Jailer)
Dept. of Medicine, St. Luke's Hospital, N. Y. City

Administration of vasopressin to non-hypophysectomized animals and man results in an increase in output of adrenocortical steroids (1,2,3,4). This effect has been attributed to stimulation of the adenohypophysis by the vasopressin which in turn results in release of ACTH or some substance with ACTH-like activity(1-6). However, Guillemin and Hearn(7) as well as Saffran and co-workers using tissue culture technics(8) were unable to confirm any adenohypophyseal stimulation by vasopressin. More recently, Royce and Sayers(9) have noted increased adrenocortical steroid secretion in hypophysectomized as well as decapitated rats receiving vasopressin and have concluded that vasopressin must release ACTH from peripheral binding sites. Because of these somewhat conflicting reports it occurred to us that vasopressin might directly stimulate the adrenal cortex and that the adrenal perfusion technic, as developed in this laboratory(10), would be an ideal method for studying this possibility.

Methods. Normal adult mongrel dogs, weighing between 15-20 kg, were anaesthetized by intravenous administration of 0.46-0.62 mg/kg body weight of sodium pentobarbital. The hypophysis was removed by transbuccal approach(11). Adrenals were then prepared for perfusion by the technic of Hilton *et al.*(10). Donor hypophysectomized dogs were bled from femoral artery the morning of experiment. This arterial blood was perfused by mechanical pump into the adrenal preparation of recipient hypophysectomized animal. Synthetic vasopressin‡ was added to arterial circuit leading to adrenal glands at rate of 1 ml/minute for 7-10 minutes. Concentrations used varied from 0.01-0.4 pressor units/ml (0.03-1.0 μg). Blood flow rate through glands was kept constant at 10 cc/minute throughout each experiment. At end of each experiment an injection of ACTH at 1 ml/minute for 7-10 minutes and at concentration of 0.5-2.5 units/ml was given to verify adrenal viability. Adrenal venous blood was collected in graduated cylinders, rate of flow was measured, and concentration of hydrocortisone in plasma was determined by the method of Peterson *et al.*(12).

Results. Ten experiments were performed at different dosage levels of both arginine and lysine vasopressin. Control secretion rates of hydrocortisone were uniformly low, averaging less than 0.5 μg/minute, in all 10 experiments. In the lysine group (4 experiments) hydrocortisone§ secretion was increased, as much as 6-fold, over control resting rates. Following ACTH administration at end of each experiment there was further increment in hydrocortisone secretion. This same pattern of response was noted in 5 experiments in which arginine vasopressin was perfused.

An attempt was made to demonstrate a dose-response curve utilizing 0.01 unit/minute, 0.1 unit/minute and 0.4 unit/minute of arginine vasopressin (Fig. 1). A marked increase in hydrocortisone secretion was observed following initial small dose and a significant further increase was noted upon administration of 0.4 units/minute.

High doses of vasopressin appeared to result in more prolonged as well as greater hydrocortisone secretion. This can also be seen in Fig. 1 where hydrocortisone secretion was still elevated 20 minutes following end of perfusion of 0.4 unit/minute of arginine vasopressin, as contrasted with the more rapid fall off in secretion rate following 0.1 unit/minute.

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† Fellow of N. Y. Heart Assn.

‡ Synthetic lysine and arginine vasopressins were kindly supplied by Vincent du Vigneaud's laboratory.

§ Spectrophotometric analysis revealed maximum absorption at wave length of 410 mμ.

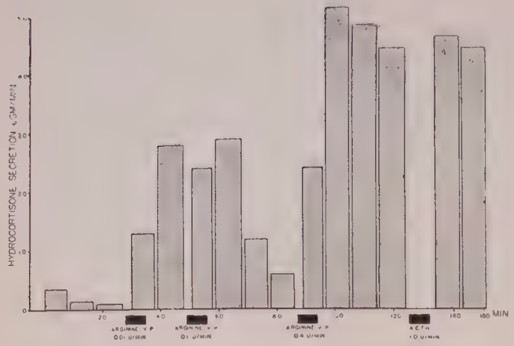

FIG. 1. Effect of graded doses of arginine vasopressin on hydrocortisone secretion from perfused adrenal glands of the dog. All substances were administered over 7 min. time interval.

The advantage of direct perfusion technic in these experiments is elimination of interference from other endocrine glands or organs. These factors may in part explain the lack of effect of vasopressin on adrenocortical secretion, noted by some investigators(13). Whole body dilution of injected vasopressin, inactivation of material by liver or at other sites prior to reaching the adrenal glands, possible species differences etc., must all be taken into account in interpretation of previously reported negative results.

The data indicate that synthetic vasopressin has a direct stimulatory effect on the adrenal glands, enhancing secretion of hydrocortisone, and appears to be in some instances as potent a stimulator as adrenocorticotrophic hormone itself.

It is evident from our studies that data which have accumulated on vasopressin stimulation of ACTH or an ACTH-like substance is not, in fact, due to such stimulation but is a direct effect of vasopressin on the adrenal cortex itself. ACTH effect in our preparation is excluded by: (1) use of synthetic vasopressin (2) use of hypophysectomized animals (3) low resting secretion rates from adrenal gland prior to vasopressin administration (4) direct perfusion of adrenal glands *via* their arterial circuit.

This surprisingly potent adrenocortical stimulating effect of vasopressin implies that it may be an important factor in reaction to stress. Our preliminary studies indicate an additive effect when vasopressin is given to ACTH-stimulated adrenal gland preparation. This raises the possibility that vasopressin stimulates adrenal steroid secretion by a mechanism which is distinct from that of ACTH.

Summary. By means of direct arterial perfusion of the adrenal glands in the dog it has been shown that synthetic vasopressin stimulates secretion of hydrocortisone. This effect is not mediated *via* the adenohypophysis or any other organ, but is rather the result of direct stimulation of the adrenal cortex by vasopressin itself.

The technical assistance of Katherine Tengerdy and Elizabeth Rezek is gratefully acknowledged.

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Effects of Diethanolamine (DEA) on Rats Fed Atherogenic Diets.* (24684)

THOMAS B. CLARKSON AND HUGH B. LOFLAND (Introduced by Camillo Artom)
Vivarium and Dept. of Biochemistry, Bowman Gray School of Medicine, Wake Forest College

Diethanolamine has been shown to affect the lipide composition of the liver, and to be incorporated into a phospholipide analogous to phosphatidyl-ethanolamine (Artom, *et al.* (1-3)). Clarkson, *et al.*(4), and King *et al.* (5) reported that diethanolamine salt of camphoric acid ester of 4,dimethylbenzyl alcohol (Gallogen®) suppressed elevation of cholesterol in tissues and serum of cockerels fed cholesterol-rich diets. Subsequent studies have suggested that DEA is the active moiety of this molecule(6,7). Quite recently, Dayton(7), using rats on hypercholesterolizing diets found that Gallogen® and DEA both depressed liver cholesterol, but increased further the cholesterol level in serum. In the present experiments rats were also used, but hypercholesterolizing diets contained a high proportion of fats with different degrees of unsaturation. When a fat low in unsaturated fatty acids (hydrogenated coconut oil) was fed, DEA reduced significantly liver lipides, liver cholesterol, aortic cholesterol, and serum cholesterol. However, these effects of DEA were not apparent when a more unsaturated fat (corn oil) was included in the diet.

Methods. Male albino rats of Wistar MW-2 strain† with initial weight 50 to 80 g, were placed on hypercholesterolizing diets similar to those described by Fillios, *et al.*(8). The exact compostion of diets is presented in Table I. After 4 weeks on experimental diets, the rats were anesthetized with ether and 1 ml blood sample obtained by cardiac puncture for total serum cholesterol. The rats were killed by exsanguination after 8 weeks on the diets. Livers were removed, weighed, and minced immediately under alcohol. Lipides were extracted with hot alcohol and alcohol-ether, and purified with chloroform(9). On aliquots of liver lipides, the weight of the chloroform extract ("total lipides"), total cholesterol(10) and lipide phosphorus(11) were determined. The thoracic aorta was removed, cleared of adhering extraneous tissue, and weighed. After mincing in alcohol, the aortas were extracted with alcohol-ether. For cholesterol determination on aorta and serum, the Abell procedure(10) was used.

Results of our determinations of serum cholesterol are presented in Table II. At the end of fourth week there were no significant differences between cholesterol levels in sera of DEA treated rats and those of corresponding controls. At this time, average level of cholesterol in serum of rats fed corn oil was not as high as in rats fed coconut oil. On the other hand, it appears that after 8 weeks, DEA was quite effective in lowering serum cholesterol, when hydrogenated coconut oil was the fat component of the diet. There was no such effect of DEA when corn oil was fed for same length of time. The discrepancy between our results and those of Dayton is possibly due to differences in level and type of fat included in the diet.

Results of analyses of liver and aorta are also shown in Table II. In both tissues, DEA lowered the cholesterol level when the diet contained coconut oil, but had no significant effect in corn oil-fed groups. In liver, total lipides were depressed by administration of

TABLE I. Composition of Diets.

Dietary ingredients*	Group No. I	II	III	IV
Hydrogenated coconut oil† (30%)	+	+	—	—
Corn oil (30%)	—	—	+	+
Diethanolamine HCl (.05%)	—	+	—	+

* Diets contained, in addition to substances listed above, vitamin free casein, 30%; sucrose, 21.2%; starch, 21.2%; salt mixture, U. S. P. XII, 4%; Alphacel (Nutritional Biochemicals Corp.), 2%; thiouracil, 0.3%; cholesterol, 1%; sodium cholate, 0.3%; and vit supplement (Vit Diet Fortification Mixture, complete, Nutritional Biochemicals Corp.), 2.2%.

† Supplied through courtesy of Dr. F. H. Mattson, Procter and Gamble Co., Cincinnati, O.

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* Supported by grants from N. Carolina Heart Assn., United Medical Fn., and U. S. Atomic Energy Comm.

† Obtained from Manor Farms, Staatsburg, N. Y.

TABLE II. Effects of Diethanolamine (DEA) Added to Atherogenic Diets.*

Group No.†	Rat body wt (g)	Aorta cholesterol (mg)‡	Serum cholesterol (mg)§		Liver lipides (mg)‖		
			4 wk	8 wk	Cholesterol	Phospholipides¶	Total lipides
I. Coconut oil (25)	181.0 ± 4.7	7.1 ± .6	537.0 ± 26.0	2220.1 ± 174.2	513.1 ± 29.1	181.3 ± 3.1	1051.2 ± 35.0
II. Coconut oil & DEA (25)	193.7 ± 5.7	4.5 ± .1	527.0 ± 29.1	1428.6 ± 128.1	264.4 ± 17.9	141.2 ± 9.1	697.0 ± 38.1
III. Corn oil (24)	200.6 ± 5.5	4.5 ± .1	442.3 ± 22.4	732.3 ± 62.2	512.3 ± 57.8	145.0 ± 8.0	980.1 ± 62.2
IV. Corn oil & DEA (18)	195.1 ± 5.4	4.9 ± .3	448.7 ± 28.3	757.0 ± 50.0	414.2 ± 42.1	163.2 ± 7.9	922.0 ± 66.0

* Avg values, followed by stand. errors of means. † No. of rats in parentheses.
‡ Values/g of aorta (wet). § Values/100 ml serum. ‖ Values/whole liver of a 100 g rat.
¶ Lipide P × 25.

DEA, but significantly only in rats fed coconut oil. In the latter animals, DEA also appeared to reduce liver phospholipides, whereas in rats fed corn oil, the results were in the opposite direction.

The present experiments shed little light on the mechanism of the action of DEA. Inasmuch as phospholipide synthesis in liver appears to be affected by this compound(1-3), it seems possible that our results reflect changes in mobilization from the liver, and/or in transport of lipides in plasma. For the present, however, any such interpretation must be purely speculative.

Summary. In rats fed hypercholesterolizing diets, DEA significantly reduced liver lipides, liver cholesterol, aortic cholesterol, and serum cholesterol, when the fat component was hydrogenated coconut oil. There was no effect of DEA when dietary fat was corn oil.

The authors are indebted to Dr. Camillo Artom for helpful suggestions, and Katherine King and Jack Dawson for valuable technical assistance.

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Bovine Ocular Squamous Cell Carcinoma. I. Tissue Culture Studies of Plaque.* (24685)

J. A. Sykes, L. Dmochowski and W. O. Russell†‡
Section of Virology and Electron Microscopy, and Dept. of Pathology, University of Texas M. D. Anderson Hospital and Tumor Inst.; and Dept of Microbiology, Baylor University College of Medicine, Houston, Texas

The pathological anatomy of bovine ocular squamous cell carcinoma has been fully described(1). This disease includes benign hyperplasia (plaque) and pappilloma, which often precede development of carcinoma. Eosinophilic intranuclear inclusion-like bodies, not seen in eyes of apparently disease-free cattle, have been described in cells of benign and malignant lesions(1). This observation, combined with bilaterality, disappearance and reappearance of the benign precursor lesions, plaque and papilloma, suggested that a virus, among other factors, may play a part in origin of this disease(1). Tissue culture studies were undertaken, as a first step, to determine whether a viral agent could be implicated in the origin of benign or malignant lesions of the disease.

Materials and methods. The first lesion to be investigated was plaque, one of the benign precursor lesions. Plaque lesions were removed under local anesthesia induced by retro-ocular inoculation of 20-30 ml of 2% procaine. The method of collection of specimens in the field which gave a high percentage of successful primary cultures was as follows: After removal, a portion of the lesion was put in Bouin's fixative, the remainder placed in screw-capped vial with Eagle's basal medium containing 20% inactivated calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and stored overnight at 4°C. The following day the tissue was minced with scissors in a 60 mm Petri dish, and treated for 20 minutes at ambient temperature with minimal volume of trypsin solution (0.25% Difco Bacto Trypsin in Ca and Mg free Hanks' B.S.S.)(2). The tissue mince, suspended in trypsin solution was then placed in 10-15 ml of complete culture medium in screw-capped test tube and stored at 4°C during transportation. Tissue fragments were resuspended in 7-14 ml of fresh medium and the suspension placed into one or more T-30 culture bottles (Kontes Glass Co., Vineland, N. J.) The cultures were left undisturbed at least 4 days. Thereafter medium changes were made every 4 days irrespective of pH. Primary outgrowth was observed to occur within 4-30 days. The cells were too thin for detailed observations by bright-field microscopy. Such observations were made possible by use of chambers specially constructed for phase microscopy(3). These chambers also allow study of the same culture after staining.

Results. Phase microscopy of cells from plaque lesions grown in the chambers, confirmed early observations made by bright-field microscopy on stained cells in T-30 flasks(4). As already reported, 2 types of cells have been observed in tissue cultures of plaque. One type of cell is large and contains one or more large nuclei; the other is considerably smaller and as a rule contains a single small nucleus (Fig. 1). Some of the larger cells may be seen at somewhat higher magnification in Fig. 2. Well marked tono-fibrils have been observed in the larger cells (Fig. 3). Characteristic cell with large cytoplasm and strongly basophilic granular material in the perinuclear region may also be seen. Cytoplasm of the larger cells has frequently been found highly vacuolated (Fig. 4). Vacuolization of cytoplasm occurs with equal frequency in larger and smaller size cells (Fig. 5). Vacuoles in the cytoplasm have often been found to contain strongly basophilic material (Fig. 6). Nuclei contained a number of nucleoli variable in size and shape, and show frequent margination of chromatin and vacuolization (Fig. 6).

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† Invaluable cooperation of ranch owners in this study is gratefully acknowledged.

‡ This is publication No. 6 of Cancer Eye Study Section.

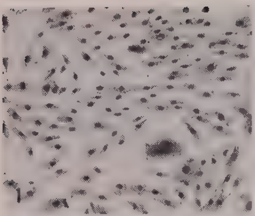
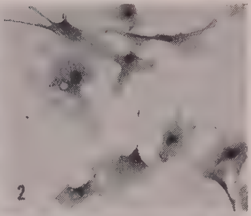
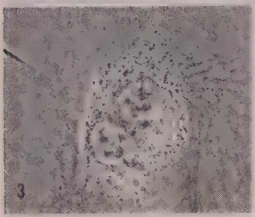
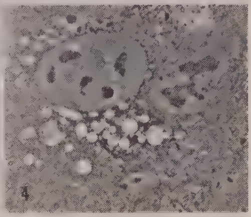
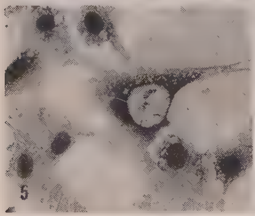
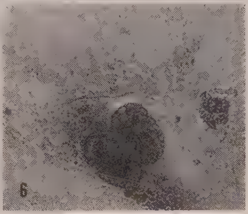

FIG. 1. Cells derived from Plaque, 21st passage, showing small and large cell types. May Grünwald-Giemsa stain. × 38.

FIG. 2. Cells similar to those shown in Fig. 1 at higher magnification. May Grünwald-Giemsa stain. × 60.

FIG. 3. Phase contrast micrograph of a large cell showing tono-fibrils and granular material in perinuclear zone. × 150.

FIG. 4. Phase contrast micrograph of perinuclear region of a large cell showing vacuolization. × 263.

FIG. 5. Vacuolated cells, one showing a large vacuole containing small discrete aggregations of basophilic material. May Grünwald-Giemsa stain. × 150.

FIG. 6. Portion of a large cell with vacuoles containing strongly basophilic granular material. May Grünwald-Giemsa stain. × 225.

Vacuolar degeneration of cytoplasm has been observed in scattered foci of cells in cultures of plaque gradually extending towards the periphery. The changes appear in cultures of fifth or subsequent passages and lead to loss of the culture even after frequent subcultures are made. Cultures of a number of plaque lesions have been maintained for more than 45 sequential passages, for more than 30 weeks. One hundred plaque lesions were put into tissue culture; 53 were maintained 1-5 weeks, 7 for 5-10 weeks, 28 for 10-20 weeks, and 5 for 20-40 weeks. Seven cultures grew for less than 1 week. Cells of 18 plaque lesions are presently in culture, 3 have been in culture for 30 weeks and 45 passages, 15 for 10 weeks and 7 passages.

Studies are in progress to determine whether the observed changes are due to a viral agent.

Summary. The method of collection and cultivation *in vitro* of specimens of plaque, one of the benign precursor lesions of bovine ocular squamous cell carcinoma, is described. Primary outgrowths were obtained in 98% of collected specimens. Cells derived from plaque lesions, following a number of consecutive passages, show nuclear and cytoplasmic changes which may appear as early as the 5th passage. These changes lead to the eventual loss of the culture. Some specimens of plaque lesions in which changes have as yet not been observed are now in the 46th consecutive passage. The nature of the observed changes is being investigated.

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Relationship of Serum Phenylalanine Levels and Ability of Phenylketonurics to Hydroxylate Tryptophan. (24686)

ROBERT C. BALDRIDGE, LEATRICE BOROFSKY, HENRY BAIRD III, FREDERICK REICHLE AND DONNA BULLOCK (Introduced by M. J. Oppenheimer)
Depts. of Physiological Chemistry and Pediatrics, and St. Christopher's Hospital, Temple University School of Medicine, Philadelphia, Pa.

Although there is evidence(1) in phenylketonurics for hereditary lack of a component of the enzyme system which catalyzes conversion of phenylalanine to tyrosine, the cause of associated mental retardation remains unknown. Increased excretion of non-phenylalanine metabolites such as indoleacetic acid and indolelactic acid by uncontrolled phenylketonurics has been reported(2), and Pare *et al.*(3) noted lower levels of 5-hydroxytryptamine (serotonin) in serum and decreased amounts of 5-hydroxyindoleacetic acid (5-HIAA) in urine of uncontrolled phenylketonurics compared with similar data for normal and non-phenylketonuric mentally retarded children. The latter workers suggested decreased formation of serotonin may be related to mental defect. The concept has been developed that increased concentrations of phenylalanine (or related metabolites) in blood and tissues of phenylketonurics may competitively inhibit the hydroxylase system which catalyzes conversion of tryptophan to 5-hydroxytryptophan, the precursor of serotonin. Decreased rate of formation of serotonin is reflected in decreased excretion of its major metabolic end product, 5-hydroxyindoleacetic acid. If such a concept is correct, patients with high blood phenylalanine levels would be expected to excrete more 5-hydroxyindoleacetic acid following transition to a low-phenylalanine diet (with concomitant decrease in phenylalanine levels). Additionally, phenylketonuric patients whose blood and tissue levels of phenylalanine are in normal range, following dietary control, should excrete greater amounts of 5-hydroxyindoleacetic acid than do uncontrolled phenylketonurics. Finally, increasing the ratio of tryptophan to phenylalanine in the diet of the latter might result in increased formation of 5-hydroxylated products. Experiments designed to test these hypotheses form the basis of this report.

Methods. Serum phenylalanine levels were determined by paper chromatographic method of Berry(4). Urinary 5-hydroxyindoleacetic acid was measured essentially as described by Udenfriend *et al.*(5). With each urine sample, parallel analyses were made of aliquots to which were added known amounts of 5-hydroxyindoleacetic acid. Recovery of the latter ranged between 80-90%. Creatinine determinations were performed by the Jaffe alkaline picrate procedure and in some cases by adsorption method (with Lloyd's reagent). Urine collection bottles contained acetic acid as a preservative and contents were frozen and stored at –20°C until analyzed 2-3 days later. Attempts were made to collect 24 hour urine specimens from each patient; however, due to uncertainties of collection, data are presented in terms of ratio of μg of 5-hydroxyindoleacetic acid excreted/mg of creatinine. The low-phenylalanine diet consisted of Lofenalac* plus foods calculated to give daily intake of 15-25 mg of phenylalanine/kilo body weight.

Results. The inverse relationship between serum phenylalanine levels and excretion of 5-hydroxyindoleacetic acid during transition to a low phenylalanine diet is illustrated in Fig. 1 (patient W). An increase in 5-hydroxyindoleacetic acid excretion occurred within 4 days following ingestion of the low-phenylalanine diet.

After he had eaten the low-phenylalanine diet for 4 months (Table I), the 5-hydroxyindoleacetic acid to creatinine ratio of patient W was increased to over 10; a similar increase was noted with a second patient (C). Other patients (D and S) who had been fed the diet for 4 to 10 months excreted amounts of 5-hydroxyindoleacetic acid which, when expressed in terms of ratio of 5-hydroxyindole-

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* Lofenalac, complete dietary preparation, was kindly furnished by Mead Johnson and Co.

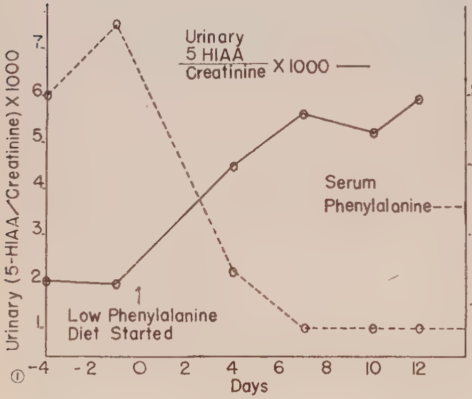
TABLE I. Relation of Diet, Serum Phenylalanine Levels and Urinary 5-Hydroxyindoleacetic Acid (5-HIAA) of Phenylketonurics.

		Prior to ingestion of low phenylalanine diet		After ingestion of low phenylalanine diet*		
Patient	Age (mo) at start of diet	Serum phenylalanine (mg %)	Urinary 5-HIAA (μg/mg of creatinine)	Period fed diet (mo)	Urinary 5-HIAA (μg/mg of creatinine) Range	Mean
W ♂	30	25	2.0	4	9.9–10.4	10.2†
C ♀	18	50	1.9	10	9.2–11.5	10.4†
D ♀	15	60		2		5.4
				10	9.2–11.1	10.2†
S ♀	25	40		2– 4	10.4–14.8	12.8‡
				7–10	11.2–13.8	11.9‡

* Serum phenylalanine levels were less than 5 mg %. † Mean of 2 determinations, 3-5 days apart. ‡ Mean of 4 determinations, 2-3 wk apart.

acetic acid to creatinine, were also in the same range.

A phenylketonuric (B, age 38 months, a first cousin of patient W) who refused to eat the low-phenylalanine diet, was fed 100 mg of L-tryptophan/kg body weight on 5th and again on 8th day after admission to hospital. There was an immediate increase in excretion of 5-hydroxyindoleacetic acid following administration of tryptophan (Fig. 2). Excretion decreased on 10th day to the lower level (ratio of 2-3) typical of uncontrolled phenylketonurics.‡ Patient B later was fed 100 mg of 5-hydroxytryptophan† daily along with normal diet. Between 10 and 15% of the amount fed was excreted in urine as 5-hydroxyindoleacetic acid (measured by Udenfriend procedure). Assessment of the value of 5-hydroxytryptophan administration must be postponed. However, preliminary testing revealed a spurt in mental development in (B) who was given the latter compound. His serum phenylalanine levels ranged from 20-25 mg % during the period studied.



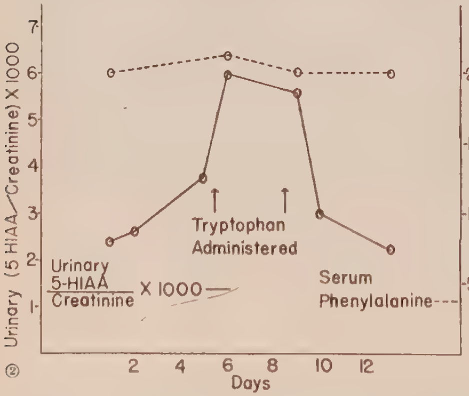


FIG. 1. Changes in serum phenylalanine levels and urinary 5-hydroxyindoleacetic acid (5-HIAA) excretion during transition to a low-phenylalanine diet.

FIG. 2. Effect of administration of tryptophan on 5-hydroxyindoleacetic acid excretion by uncontrolled phenylketonuric.

Discussion. Patients studied by Pare *et al.* (3) were older than those described here. However, urinary 5-hydroxyindoleacetic acid/creatinine ratios reported for their group were similar to those noted in our patients prior to dietary control. After present studies had been completed, Pare's group reported(6) that phenylketonurics serum serotonin levels were significantly higher after ingestion of a low-phenylalanine diet for 4 weeks than before dietary control. Their failure to note significant differences in excretion of 5-hydroxyindoleacetic acid in the time studied, was attributed to use of random rather than 24-hour samples of urine. Ages of patients studied were not given. While our studies were in progress, Berendes *et al.*(7) reported that phenylketonurics who had been fed low-phenylalanine diets excreted more 5-hydroxyindoleacetic acid/24 hours than did untreated phenylketonurics. Creatinine values were not given.

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‡ The feeding of equivalent amounts of L-tryptophan to two phenylketonurics who had been eating a low phenylalanine diet (serum phenylalanine levels less than 5 mg %) was *not* followed by such an increase in 5HIAA excretion.

† We are indebted to Dr. Gustav J. Martin, National Drug Co., for supplies of 5-hydroxytryptophan,

Summary. 1. Following a decrease in serum phenylalanine levels (induced by feeding of a low-phenylalanine diet) phenylketonurics excrete increased amounts of 5-hydroxyindoleacetic acid. 2. The ratio of urinary 5-hydroxyindoleacetic acid to creatinine is about 5-fold greater for young phenylketonuric children, fed a low-phenylalanine diet for 4-10 months, than for untreated phenylketonurics. 3. Oral administration of L-tryptophan to untreated phenylketonuric was followed by increase in 5-hydroxyindoleacetic acid excretion. 4. Following oral administration of 5-hydroxytryptophan, 10-15% was excreted as urinary 5-hydroxyindoleacetic acid by an otherwise untreated phenylketonuric.

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Production of Leucocytic Exudates in Rat Granuloma Pouch.* (24687)

GUILLERMO SOBERON† AND PHILIP P. COHEN
Dept. of Physiological Chemistry, University of Wisconsin, Madison

A relatively simple procedure for producing inflammatory exudates rich in leucocytes, based on rat granuloma pouch technic of Selye *et al.*(1,2,3) is reported in this paper. Several technics for production of sterile inflammatory exudates have been reported. Opie(4,5,6,7) used aleuronat and starch for production of sterile exudates in the pleural and peritoneal cavities of dogs; Ponder and MacLeod(8) employed intraperitoneal saline injection method in rabbits. Menkin(9) employed turpentine injections for production of sterile pleural exudates in dogs. Technics for separation of leucocytes from whole blood have been reported by Tullis(10). The granuloma pouch technic as developed by Selye and coworkers for study of anti-inflammatory agents(3) appeared to us to offer several advantages for the purposes of our studies. Conditions necessary for producing an inflammatory exudate rich in myeloperoxidase-containing polymorphonuclear leucocytes are here reported.

Methods. Male, Sprague-Dawley rats were used. *Formation of pouch.* After ether anesthesia, the back of the animal was shaved with fine electric clippers and 1% iodine solution applied on the skin. Forty to 60 ml of air were introduced subcutaneously by means of a syringe and 22 gauge needle. The pouch usually covered about ¾ of the back of the animal. Care must be taken at time of insufflation to prevent air from going into the forelegs and cervical region. This can be avoided by putting pressure on the skin with the free hand. The irritant was introduced immediately after the pouch was formed. *Collection and nature of cells in exudate.* The fluid formed in the pouch was extracted with a syringe attached to 22 gauge hypodermic needle. The collected exudate was centrifuged at room temperature and leucocytes washed 3 times with 0.9% saline. Wright stain(11) and benzidine stain(12) were used to differentiate the cells and to evaluate the myeloperoxidase content. Cell counts were made with hemocytometer(13). *The following aspects were evaluated*: a) size of pouch, b) regularity and shape of the pouch, c) volume of exudate, d) nature of exudate, e) number of leucocytes, f) per cent neutrophils, g) myeloperoxidase content. Various factors that might affect cellular composition of the exudate were also studied. The pouch was always formed in the same general way and only one of the factors was varied in a group of rats at any given time. Groups were from 5 to 10 animals. The factors investigated were 1) irritant used, 2) number and type of injections of turpentine, 3) size of animal, 4) local pH and 5) time of withdrawal of fluid.

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† Fellow of E. R. Squibb and Sons, Mexico, S. A. Present address, Dept. of Biochemistry, Hosp. de Enfermedades de la Nutricion, Mexico City. D.F.

Results. Although there was some individual variation the findings with each group are sufficiently reproducible to permit evaluation of the factors under consideration.

1) *Effect of irritant used.* a) Croton oil at 0.5% (v/v) concentration in corn oil consistently produced hemorrhagic exudates witn relatively few leucocytes of low myeloperoxidase content. b) Peptone culture broth produced no significant amount of exudate. c) Turpentine (commercial preparation) gave good results. The volume of exudate varied from 4 to 10 ml and the purulent fluid contained 60 to 80% neutrophils with high myeloperoxidase activity.

2) *Number and type of injections of turpentine.* Three-tenths ml of turpentine was introduced on first and fifth day, and fluid withdrawn on seventh day. When only one injection of turpentine was used no fluid was detected in the pouch. A second injection on the day previous to withdrawal was effective in producing the exudate. When this second injection was applied between fourth and sixth day after first injection, consistently good results were obtained, but when it was applied after the sixth day, only a small amount of exudate was found (1 to 3 ml) and percentage of neutrophils and myeloperoxidase content were low.

In groups given single injections of 0.5 ml of 5%, 10% and 50% turpentine solution in corn oil, the amount of exudate was small. Percentage of neutrophils was high but myeloperoxidase activity was low.

One group injected with 0.3 ml of turpentine alone showed good results, but the pouches were quite irregular. The best results were obtained when rats were injected with 10 ml of 3% (v/v) suspension of turpentine in 0.01 M phosphate buffer pH 7.0. One percent solution of Alconox was added (1 ml to each 9 ml suspension) and the suspension made homogeneous by shaking immediately before injection. A suspension of Alconox and buffer without turpentine had no effect.

3) *Effect of size of rats.* Rats over 250 g were not found suitable for formation of regular pouches. Best results were observed with groups of rats weighing between 150-200 g. In a group of small rats (100-125 g) 7 out of 10 died on day the pouch was formed. Good results were obtained in survivors. 4) *Effect of local pH.* (Table I) Five groups of rats received turpentine suspended in 0.01 M phosphate buffer at pH's of 6.0, 6.5, 7.0, 7.5 and 8, in groups 1 to 5 respectively. Three out of 10 animals died in the first group and 6 out of 10 in the fifth. No difference was observed in nature of exudate formed in rats in Groups 2, 3, 4 and survivors in Groups 1 and 5.

5) *Effect of time of withdrawal of fluid.* When the exudate is obtained 24 hours after second injection of turpentine, the percentage of neutrophils is high (90-100%). After 48 hours the percentage of leucocytes was 70-90 and when 3 days elapsed after second injection the neutrophil percentage dropped to 50-

TABLE I. Effect of pH of Turpentine Suspension on Leucocyte Production, in 5 Groups.

No. of rats	pH of turpentine suspension	Nature of exudate	Vol (ml) Range	Vol (ml) Avg	No. of cells × 10^{-3} Range	No. of cells × 10^{-3} Avg	% neutrophils	MPO content	pH of exudate
10*	6.0	7 purulent	4-7	5	15-25	18	90-100	3+ to 4+	
9	6.5	8 purulent 11 hemorrhagic	5-9	6	10-28	20	90-100	*Idem*	7.0 to 7.2
7	7.0	7 purulent	3-9	6	12-22	16	90-100	"	
8	7.5	7 purulent 1 hemorrhagic	5-8	5.5	15-25	19	90-100	"	7.0 to 7.2
10†	8.0	4 purulent	4-8	5	9-23	14	90-100	2+ to 4+	

* Three animals died in Group 1. † Six animals died in Group 5.

Animals inj. with 3% turpentine suspension in 0.01 M phosphate buffer pH as indicated in column 2, plus Alconox. First inj. made immediately after pouch was formed; second inj. made 5 days later. Exudate was collected on day following second inj.

60. Repeated injections of turpentine suspension gave satisfactory yields of leucocytes. Thus, if a second injection is made on fifth day after pouch is formed, and the exudate collected on sixth day, a third injection (20-30 ml of turpentine suspension) on ninth day resulted in a good yield on the tenth day. As many as 5 injections have been made in groups of rats with good yield of exudate resulting on day following. With successive injections of turpentine suspensions, at pH 7.0, it is possible to realize an exudate of 5-10 ml/rat, each time with a count ranging from 10-30 x 10^3 leucocytes mm^3, and a high neutrophil percentage (90-100). The myeloperoxidase content was consistently high in these exudates. Turpentine suspensions older than 2 weeks turn yellow and are highly toxic. The suspension should be stored in a brown bottle in the cold and prepared fresh each week. The exudate usually has a layer of turpentine floating on top after centrifugation, which is readily removed by decantation.

Evolution of the pouch. The first injection of the irritant causes formation of a granulomatous membrane apparent by the fourth day. The second injection of the irritant initiates appearance of exudate in the pouch. By seventh day the pouch begins to shrink and becomes quite small by sixteenth to seventeenth day. By the twentieth to twenty-second day the pouch collapses leaving a fibrous scar. In most animals a small necrotic area of skin is observed. There is, however, complete recovery, and it is not possible to distinguish treated animals after 5 or 6 weeks from normal animals.

Discussion. The granuloma pouch technic presented has proved most satisfactory for producing an exudate which contains relatively large quantities of myeloperoxidase-rich leucocytes in the rat. Gross contamination with erythrocytes was only occasionally experienced.

No difference in effect of local pH as far as cellular composition of exudate is concerned was observed. Control of this factor, however, was limited to adjustment of pH of the turpentine suspension. This criterion has questionable validity, however, since the pH of exudates was between 7.0 to 7.2, when pH of the injected suspension varied between 6.5-7.5. How soon the adjustment of pH is effected inside the pouch is not known. We were interested in the effect of this factor from reports of Menkin *et al.*(9,14,15,16) who state that pH has a marked influence on cellular composition of the exudate with local acidosis favoring disappearance of polymorphonuclear leucocytes. Harris(17) has challenged this view, stating that from present evidence no connection between pH and composition of inflammatory exudates has been established. Menkin(18) has answered Harris' criticism by stating that the different results might be due to differences in material under study, since leucocytes from several animal species behave differently in several aspects. Studies in rabbits by Harris(19) failed to show any correlation between pH and cellular sequence of the exudate. The guinea pig seemed to show a slightly better correlation (20). To our knowledge systematic studies on this aspect have not been done with the rat.

Another controversy has arisen concerning use of turpentine to produce leucocytic exudates. Harris(19) reported it to be toxic to cells. Menkin(18) explains his different observations as due to use of different species. We can only say that in our experiments the leucocytes in the exudate which results from injection of turpentine in the granuloma pouch showed high myeloperoxidase activity after being washed with saline.

Summary. The rat granuloma pouch has been found to be a convenient source for collection of leucocytes rich in myeloperoxidase. Of a number of irritants tried, a suspension of turpentine was most satisfactory. Within a limited range, pH of turpentine suspension had no significant effect on cellular composition of the exudate.

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Effects of Dietary Fat upon Polyunsaturated Fatty Acids of Blood in Patients with Multiple Sclerosis.*† (24688)

B. GERSTL, W. E. DAVIS, JR., J. K. SMITH, E. ATHINEOS AND G. HEROLD
Vet. Admin. Hosp., Oakland, and Dept. of Pathology, School of Medicine,
University of California, San Francisco

Among several hypotheses advanced concerning the etiology of multiple sclerosis (MS), 2 schools of thought have recently gained prominence; one(1) proposes that an allergic reaction is involved; the other(2,3) that an enzymatic or metabolic defect underlies recurrent attacks of demyelination. We reported previously that some patients with MS revealed higher plasma levels of dienoic acid than did controls(4). This observation, together with the dietary studies of Swank(5) and increased chylomicron index noted by Skillen, *et al.*(6), warrants further investigation of the lipid metabolism in this disease.

Methods. The plan of this study was similar to that of Holman, *et al.*(7). Diagnosis of MS was established as outlined previously (4). A baseline blood sample was obtained after patient had been on average hospital diet including approximately 20% protein, 28% fat, and 52% carbohydrate. Then, all individuals received a nearly eucaloric diet with soya bean oil as main source of polyunsaturated fatty acids (PUFA). Composition of diet, its lipids and vitamins added, is given in Tables I-A, B and C. Diagnoses of control patients are listed in Table II. Sequence of

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† Presented, in part, before Am. Inst. Biol. Sci., Stanford Univ., August 29, 1957.

TABLE I-A. PUFA-Enriched Diet Based on Approximately 2600 Calories/Day Intake.

	g
Non-fat milk solids	149.4
Soya bean oil	71.5
Lecithin (Granulestin)	52.7
2½ slices of bread	42.6
Gelatin	150.0
Glucose	31.2

Coffee, tea, green salad, raw carrots, radishes and celery *ad lib.*

TABLE I-B. Lipids of Diet in g (Avg of 3 Analyses).

	Soya bean oil	Granulestin
Total grams	71.5	52.7
Fatty acids:		
Unsaturated: mono-	20.2	10.8
di-	38.6	25.4
tri-	3.6	2.4
Saturated	6.0	6.7

TABLE I-C. Vitamins/Day.

Methionine	1 g
Pyridoxine	50 mg
Pentothenate	9 "
Nicotinamide	90 "
B_{12}	3 μg
Riboflavin	39 mg
Thiamine	36 "
Ascorbic acid	225 "
B-2	1,200 units
A	15,000 "

patients was kept alternating to exclude influence of seasonal factors. Blood was obtained after overnight fasting. Clotting of blood was prevented by addition of dry potassium oxalate. The cells were rapidly separated by centrifuging at 3,000 rpm. Plasma was extracted and total lipids and cholesterol determined as in previous investigation(4). The PUFA were measured by method of Herb and Riemenschneider(8). Precautions recommended by Holman(9) were followed. Measurement at 374 mμ was included, and the PUFA calculated according to Hammond and

TABLE II. Five Control Cases.

Race	Age	Diagnosis
White	21	Rheumatic fever
Negro	36	Epilepsy
"	47	Traumatic paraplegia T-4
White	31	
"	34	Epilepsy

TABLE III. Effects of Soya Bean Oil Diet upon Plasma Polyunsaturated Acids.

	Diet	No. of individuals		Chol. / Lipid	PUFA / Lipid	Diene / PUFA	Triene / PUFA	Tetraene / PUFA	Di/Tri*	Tri/Tetra*
						%			Ratio	
MS patients	Free	6	Avg	26.2	15.8	62.3	13.1	16.0	5.16	.84
			Range	19.7–32.6	7.8–20.2	59.5–68.5	8.2–17.4	12.9–18.7	3.43– 8.38	.50–1.35
	PUFA (1 wk)	5	Avg	21.0	20.9	72.4	13.0	11.5	6.62	1.13
			Range	18.1–23.4	17.4–24.1	66.1–78.1	6.7–25.3	7.7–16.4	2.76–11.32	.55–1.54
	" (3 ")	4	Avg	21.9	20.7	70.7	13.9	9.6	5.49	1.49
			Range	10.8–27.7	18.3–23.9	66.7–79.7	8.8–17.9	7.7–11.7	3.82– 9.06	1.08–2.25
Controls	Free	5	Avg	25.3	16.0	61.1	13.9	16.5	5.11	.89
			Range	20.2–28.7	13.9–18.5	50.4–75.8	8.1–20.2	10.5–23.9	2.96– 9.40	.54–1.47
	PUFA (1 wk)	5	Avg	24.6	21.5	71.3	12.2	10.1	6.73	1.23
			Range	17.1–36.7	15.5–24.0	59.0–82.1	7.6–18.6	6.0–13.3	3.17–10.00	.69–1.71
	" (3 ")	4	Avg	22.4	21.8	74.8	10.2	9.8	8.79	1.06
			Range	18.8–24.7	17.5–25.5	69.8–83.9	5.2–14.6	6.5–13.0	4.79–16.25	.71–1.65

* Ratios calculated individually and then averaged.

TABLE IV-A. Average Difference from Baseline to 3rd Week of Diet.*†

	Control group		MS group	
Ratios	No.¶	Diff.	No.¶	Diff.
Chol./Lipid	4	–4.20‖	4	–5.40
PUFA/Lipid	4	5.35	5	5.34§
Diene/PUFA	4	10.95‖	5	9.70§
Triene/PUFA	4	–3.95§	5	– .12
Tetraene/PUFA	4	–4.77‖	5	–6.32‖
Diene/Triene	4	3.39‡	5	.98
Triene/Tetraene	4	.09‡	5	.58§

* We are indebted to Mr. R. A. Schindler for assistance in statistical evaluation.

† Values with ref. marks (‡ § ‖) are significantly different from zero at levels indicated. (Two-tailed t test for correlated means.)

‡ $p < .10$ § $p < .05$ ‖ $p < .01$

¶ No. of individuals.

Lundberg(10). Separation and estimation of PUFA in red blood cells were carried out as described elsewhere.

Results. According to Holman, *et al.*(7), ratios of individual lipids to total lipids and those between the 2 groups of structurally related PUFA are more revealing than data on individual lipids. In agreement with this concept, our results are likewise reported as ratios.

Plasma lipids. Cholesterol/lipid ratio declined and PUFA/total lipid ratio increased in both MS and control groups during 3 weeks of PUFA-enriched diet (Table III). The diene/PUFA ratios showed a rise, and tetraene/PUFA ratios showed a fall in both groups during the study, but a statistically significant difference in degree of change was not observed in either case (Table IV). The triene/PUFA ratio declined in the control group while it remained practically unchanged in MS patients (Table III), resulting in a statistically significant difference (Table IV-B). Similarly significant was the difference in behavior of the diene/triene and of the triene/tetraene ratio (Table III). The former rose markedly in the control group and only slightly in MS patients; conversely, triene/tetraene ratio increased considerably in MS, but only to a minimal extent in control patients.

Red blood cells. Because of scarcity of data available on lipids of red blood cells, these are reported in Table V. The diene/PUFA ratio remained nearly stable in MS patients and rose slightly in the controls. Triene/PUFA and triene/tetraene ratios increased in MS patients while declining in the controls; but none of the differences were statistically significant.

Discussion. This investigation of multiple blood lipid components was limited to a small number of patients, as in the study of Holman (7). Nevertheless, analysis of data obtained on our 2 groups allows certain tentative interpretations.

Cholesterol/lipid ratio decreased in both groups during the period of special diet. This corroborates the findings of Kinsell, *et al.* and of Ahrens, *et al.*(11,12). Increase of PUFA/lipid ratio was of similar extent in both groups, indicating that there was no defect in absorption of dietary fat in the MS group.

Dienoic/PUFA and trienoic/PUFA ratios in MS and control groups were almost identical at start of experiment. During the period of PUFA-enriched diet, the diene/PUFA ratio rose significantly in both groups (Table IV-A). The triene/PUFA ratio, however, declined significantly in the controls during this period, while remaining stable in the MS group, resulting in a significant dfference between the 2 groups (Tables IV-A and B). The tetraene/PUFA quotient also was alike at the start, and decreased to the same extent of p in both groups. The diene/triene as well as the triene/tetraene ratios were almost identical in the 2 groups before receiving the diet (Table III). The former increased significantly in the controls leading to a difference

TABLE IV-B. Significance of Difference between Means of MS and Control Groups.

Diet	Chol./Lipid	PUFA/Lipid	Diene/PUFA	Triene/PUFA	Tetraene/PUFA	Diene/Triene	Triene/Tetraene
Free	N.S.*	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
PUFA (3 wk)	"	"	"	.10	"	.10	.10

* N.S. = Not significant.

TABLE V. Effects of PUFA-Enriched Diet upon Red Blood Cell PUFA.

Diet			PUFA/Lipid	Diene/PUFA	Triene/PUFA	Triene/Tetraene
MS patients	Free	Avg	12.5	29.0	23.5	.77
		Range	10.3–14.5	25.5–35.4	15.7–31.2	.42–1.09
	PUFA (3 wk)	Avg	12.1	30.5	25.5	.87
		Range	10.4–15.7	29.0–33.0	19.1–30.4	.58–1.17
Controls	Free	Avg	13.5	29.5	21.9	.68
		Range	12.6–15.7	24.4–32.6	19.4–25.6	.62– .78
	PUFA (3 wk)	Avg	14.3	33.9	18.7	.63
		Range	12.5–16.6	29.3–38.4	10.5–26.8	.30– .92

with the MS group with the significance $p < 0.10$. Conversely, the triene/tetraene ratio rose in the MS group with the final difference being also $p < 0.10$.

Comparison of data presented here with those reported by Evans(13) and by Pikaar, *et al.*(14) is of but limited value since dietary intake of the latter 2 groups was not given. Diene/and tetraene/PUFA ratios in the "fasting normal" of Pikaar's group are close to ours, all other diene levels in these reports being slightly higher. A larger difference is noticeable in the triene/PUFA quotient, which is higher in our group than in the 10 fasting normals of Pikaar. Since the methods employed were identical(13) or were similar (14), a different food intake may well be responsible.

More illuminating is comparison with Holman, *et al.*(7) data. The triene/PUFA ratios in Holman's group were initially lower but, after feeding hydrogenated cocoanut oil (HCO) rose higher in 2 of their subjects than in any of ours. The tetraene/PUFA ratio, under the influence of diet, declined in all our patients, as it did in 3 out of 4 of Holman's subjects receiving corn oil. The triene/tetraene ratio at start of experiments, of same range in both Holman's and our groups, rose in the MS patients to the degree observed by Holman, *et al.*, after feeding HCO.

An increase of triene/tetraene quotient has been discussed by Holman(7) as possibly signifying a PUFA deficiency. In view of abundant supply of PUFA and of similar tendency of diene/PUFA ratio in both groups, a deficiency seems unlikely. The decline of both triene/ and tetraene/PUFA ratios in the controls suggests a withdrawal of the higher PUFA from plasma when there is a plentiful exogenous supply of dienoic acid. In MS patients, however, this mechanism seems to be effective only for tetraenoic acid. The results, further, emphasize Holman's conclusion that single samples of plasma do not allow evaluation of the nutritive status of an individual as pertaining to PUFA.

Findings of red blood cell lipids did not reveal any significant changes in the ratios, though reflecting to a slight degree the changes in plasma lipids.

Summary. 1. Effects of diet providing adequate protein and vitamin intake and enriched with PUFA were studied in a group of patients with multiple sclerosis and compared with those in control patients. 2. A decline of cholesterol/lipid and of tetraene/PUFA, and an increase of PUFA/lipid as well as diene/PUFA ratios were observed in both groups. 3. Differences between the 2 groups are represented by significant decline of triene/PUFA ratio in controls and, conversely, by a rise of triene/tetraene ratio in the MS group.

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Effect of Digitalis on Potassium Toxicity in Isolated Turtle Heart. (24689)

HARRY H. LEVEEN, BENJAMIN SCHATMAN, HAROLD MOSKOWITZ
Depts. of Surgery of Vet. Admin. Hospital, Brooklyn, N. Y., and
State University of N. Y. College of Medicine

It is well accepted that potassium administration can antagonize toxic effects of digitalis(1). Patients receiving digitalis may exhibit digitalis toxicity when their potassium intake is restricted, and patients who are overdigitalized frequently respond to administration of potassium. The converse of these situations has not been satisfactorily investigated. Does digitalis antagonize the effects of potassium toxicity on the myocardium? A perfusion experiment in the isolated turtle heart was devised to study the effects of rapidly acting digitalis preparations on induced potassium intoxication. According to Szent-Gyorgi, the mineraloid hormone of adrenals abolishes "Treppe" in the isolated perfused turtle heart as does digitalis(2,3). The protective action of water soluble desoxycorticosterone glucoside against potassium toxicity was also investigated.

Methods. Hearts were removed from 30 unanesthetized turtles to include 1 cm of the truncus. The dorsal aorta was cannulated and the myocardium perfused through the coronaries, as shown in Fig. 1, with various test solutions(4). The phrenum was attached by suture to metal bar which acted as an isometric lever. Tension developed in the bar was transformed into electrical energy through suitable transducer and recorded on oscillograph. The basic solution (Howell's solution) for perfusion contained the following constituents: NaCl 0.7%, KCl 0.3%, $CaCl_2$ 0.026%, $NaHCO_3$ 0.003%, MgCl 0.01%, glucose 0.1%. Potassium was added to basic solution to augment the final concentrations of potassium by 4, 6, 8, and 12 meq/liter of potassium chloride. The substances to be tested, lanatoside C, acetyl strophanthidin and desoxycorticosterone glucoside were added in varying concentrations to the potassium enriched perfusion medium. Lanatoside C and acetyl strophanthidin were added to the perfusion media to give following concentrations: .4, .8, 1.2, and 1.6 mg/liter. Between each experiment with potassium enriched solution the heart was permitted to return to normal beat by thoroughly perfusing with plain Howell's solution.

Results. A slow infusion of KCl in Howell's solution was capable of producing cardiac arrest in most experiments when the concentration of KCl was augmented by 8 meq/l. There was individual variation, and occasionally arrest was produced with additional 6 meq of potassium/liter. Similarly, some hearts did not arrest even with an additional 10 meq/l. In lower concentrations, KCl impaired development of tension by the myocardium but did not cause arrest. Both amplitude of isometric contraction and rhythmicity were recorded. Potassium solution caused linear decrease in development of tension until asystole occurred (Fig. 2a). Cardiac rate remained constant during perfusion with potassium. Changes in rhythmicity were not progressive as were changes in amplitude of contraction. Rhythmicity was sud-

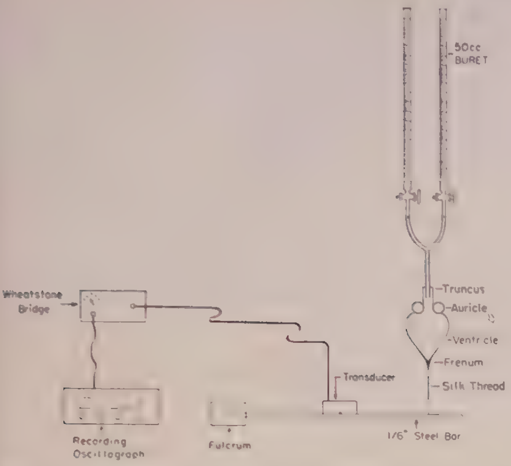

FIG. 1. Schematic diagram of technic used for recording rate and amplitude of contraction in isolated turtle heart.

denly abolished at critical concentration of potassium in the perfusate.

Addition of cardiac glycoside to the potassium containing perfusate usually resulted in sudden abolition of rhythmicity when the amplitude of contraction was not appreciably diminished. Often, complete asystole occurred after 8 or 9 contractions when the concentration of glycoside was greater than .4 mg/liter. Digitalis maintained the strength of contraction even with high concentrations of potassium (Fig. 2b). Rhythmicity was abolished suddenly even when strength of contraction was unimpaired. Increasing the concentra- of glycoside failed to protect against development of potassium asystole and even favored its more rapid development. Use of digitalis as a perfusate prior to perfusion with KCl-digitalis solution did not offer any protection against potassium arrest. However, after perfusion with KCl solution decreased the amplitude of contraction, digitalis was effective in returning the amplitude of contraction to normal. This occurred even though digitalis would cause a sudden cessation in systole. This effect on rhythmicity was partly caused by the production of heart block which could be detected visually. Desoxycorticosterone gluconate was relatively ineffective as compared to digitalis.

Discussion. Perfusion of isolated turtle heart with varying solutions of potassium was effective in first diminishing the strength of isometric contraction and eventually producing asystole. Digitalization of the isolated heart was effective in sustaining development of tension by the myocardium but did not prevent, and even accentuated, the adverse effect of potassium on spontaneous rhythmicity. The adverse effect of digitalis on rhythmicity was partially due to more rapid development of total heart block as observed by persistent auricular rhythmicity with ventricular asystole.

Although conclusions drawn from perfusion experiments in the isolated heart are hazardous, results seem to indicate that digitalis might be effective in protecting against adverse circulatory effects of hyperpotassemia in the intact animal. Digitalis exerts its action by maintaining the strength of myocardial contraction(5). Even though digitalis did not offer protection from development of asystole, the heart block is easily produced in turtles and for this reason, the situation as to rhythmicity may not be analogous to intact mammalian hearts. However, it is difficult to study development of tension in either isolated or intact mammalian hearts, and our study obviates the problem.

Summary and conclusions. Potassium causes diminished strength on isometric contractions in the isolated turtle heart. This decrease in strength of contraction is progressive with increasing concentration of potassium in the perfusate. Digitalis does not protect against development of an asystole in the isolated turtle heart, but does sustain the

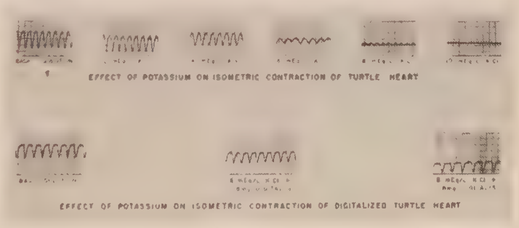

FIG. 2. Development of tension in isometric contraction of turtle heart. Strength of contraction is markedly diminished by addition of potassium to basic solution as shown in upper frames. In the lower frames, the effect of digitalis in preventing this diminution of contraction is demonstrated. At 8 meq/l of additional potassium, digitalis has sustained contraction.

strength of myocardial contraction.

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Ability of Some Progestational Steroids to Stimulate Male Accessory Glands of Reproduction in the Rat. (24690)

STANLEY C. LYSTER, G. H. LUND, W. E. DULIN
AND R. O. STAFFORD
Dept. of Endocrinology, The Upjohn Co., Kalamazoo, Mich.

The principle of multiplicity of actions of steroidal hormones has long been recognized. Steroids thought of as "estrogens" because of ability to stimulate female reproductive tract may also be "androgens" because of ability to stimulate the male tract. Similarly, hormones thought of primarily as adrenal corticoids have ability to produce progestational changes in the uterine endometrium. Progesterone resembles "androgens" because of its property of stimulating the prostate of castrated animals(1). This fact has recently assumed practical significance because of the finding of Wilkins *et al.* that occasionally women treated with progestational substances during pregnancy deliver mildly masculinized female infants(2). Some steroids recently introduced into therapeutic use are remarkably potent in causing glandular proliferation of endometrium, inhibiting ovulation, and to greater or lesser degree maintaining pregnancy in ovariectomized animals(3,4,5). Because of the possible importance of "androgenic" properties of progestins in treatment of pregnant women, a comparison has been made of ability of several of these compounds to stimulate the reproductive tract of castrate male rat. It is not known whether a substance promoting sex gland growth in the male rat will promote differentiation of the human female tract in a male direction, but the data are offered with the belief that a parallelism between the actions on these different end-points may exist.

Methods. Steroids. The drugs, together with some of their synonyms and abbreviations used in this communication, are listed below: 1. 17-α-methyl testosterone, methyltestosterone, (MT), 2. 6α-methyl-17α-acetoxy progesterone, medroxyprogesterone acetate, (MAP)*, 3. 17α-ethinyltestosterone, ethisterone, anhydrohydroxy progesterone, pregneninolone, (ET)†, 4. 17α-ethinyl-19-nortestosterone, norethindrone, (ENT)‡, 5. A mixture of norethynodrel (17α-ethinyl-5, 10-estrene-17β-ol-3-one) and 17α-ethinylestradiol, 3-methyl ether, (EN)§, 6. 17α-acetoxy progesterone, (AP)‖, 7. Progesterone (P), 8. 17α-(2-methallyl) - 19-nortestosterone, SC-9022, (MNT), 9. Testosterone propionate (TP). For administration all compounds were suspended or dissolved in cottonseed oil. *Animals.* Male rats of the Upjohn colony (Sprague-Dawley ancestry) were castrated between 26 and 28 days old. On day following castration steroid treatment began. The rats were divided into groups of 10, each group being treated with single daily oral (0.2 cc) or subcutaneous (0.1 cc) doses of cottonseed

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* *Provera,* Registered trademark, The Upjohn Co., Kalamazoo, Mich.

† *Pranone,* Registered trademark, Schering Corp., Bloomfield, N. J.

‡ *Norlutin,* Registered trademark, Parke, Davis & Co., Detroit, Mich.

§ *Enovid,* Registered trademark, G. D. Searle & Co., Chicago, Ill.

‖ *Prodox,* Registered trademark, The Upjohn Co., Kalamazoo, Mich.

TABLE I. Stimulation of Male Sex Accessory Organs by Progestins and Androgens Administered Orally.

Compound	Dose (mg/day)	No. rats	Seminal vesicle wt (mg)	Prostate wt (mg)
Vehicle only		20	6	8
Ethinyl testosterone	1	10	9	23
	2	"	10	29
	4	"	14	40
	8	"	22	50
Ethinyl-19-nortestosterone	1	"	10	13
	2	"	14	18
	4	9	18	26
Enovid	1	10	15	12
	2	"	17	17
	4	"	18	21
Progesterone	5	"	8	29
	10	"	7	28
17-acetoxy progesterone	2	"	7	15
	4	"	6	11
	8	"	6	9
	16	"	6	9
6-methyl-17-acetoxy progesterone	1	"	7	14
	2	"	7	"
	4	"	8	"
Methyl testosterone	1	20	16	48
	2	"	30	67
	4	"	87	116

TABLE II. Stimulation of Male Sex Accessory Organs by Progestins and Androgens Given Subcutaneously.

Compound	Dose (mg/day)	No. rats	Seminal vesicle wt (mg)	Prostate wt (mg)
Vehicle only		25	7	9
Ethinyl testosterone	.25	10	14	40
	.5	"	33	58
	1.0	"	44	75
	2.0	"	57	84
Ethinyl-19-nortestosterone	.25	"	13	17
	.5	"	18	24
	1.0	"	30	36
Enovid	.25	"	17	13
	.5	"	20	15
	1.0	"	19	"
Methallyl-19-nortestosterone	.25	5	6	10
	.5	5	7	13
	1.0	5	8	14
6-methyl acetoxy progesterone	.25	10	9	16
	.5	"	12	22
	1.0	"	16	25
Testosterone propionate	.01	5	13	40
	.02	5	34	62
	.025	20	48	78
	.04	5	67	90
	.05	20	84	108
	.10	"	126	146

oil alone or with steroid. Animals receiving subcutaneous treatment were sacrificed the day following tenth injection and orally-treated rats the day following ninth dose. Weights of seminal vesicles and ventral prostates were recorded at autopsy.

Results. Tables I and II present organ weights of rats treated orally with MT and 6 of the progestins and subcutaneously with TP and 5 of the progestins.

Table III ranks estimated potencies of the 7 compounds studied with respect to the 2 routes of administration and the 2 end-points. When "activity" is estimated to be <1% as effective as standard androgen, "v. sl." is entered.

TABLE III. Progestins Ranked by Order of Potency in Stimulating Androgen Target Organs.

	Oral efficacy		Subcut. efficacy	
Compound	Seminal vesicle	Prostate	Seminal vesicle	Prostate
Ethinyl testosterone	3	1	1	1
Ethinyl nortestosterone	2	2	2	v.sl.
Progesterone	v.sl.	3		
Enovid	1	4	3	"
Acetoxy progesterone	0	5		
Methyl acetoxy progesterone	v.sl.	6	v.sl.	"
Methallyl nortestosterone			0	"

Figures indicate potency in descending order, *i.e.*, most potent steroid in a series is designated "1." Thus, of the compounds showing measurable oral prostate-stimulating ability, methyl acetoxy progesterone is least potent. When potency is so slight as to be judged less than 1% of standard androgen, it is indicated as "v.sl."

Although some androgenicity is seen with most of these progestins, none of them has "androgenic" activity approaching the potency of standard androgens. In most instances androgenic actions of the progestins are so weak that their potencies cannot be compared with those of standard androgens. In general, however, they can be compared with one another. For example, the greatest prostate response to ENT is a 26-mg gland at dose of 4 mg. This is much less than that with the lowest dose of MT, so an estimate comparing its activity with MT cannot be made. But since the response falls between 23-mg gland produced by 1 mg of ET and the 29-mg gland of 2 mg of ET, with respect to prostate stimulation, ENT is greater than 25% and less than 50% as active as ET.

MAP administered *orally* showed no activity on seminal vesicle and very slight effect on the prostate. The prostate response did not increase as the dose increased, so a potency estimate would have little meaning. By *subcutaneous* route this compound showed measurable activity by both end-points, being approximately one-fourth and one-tenth ET on seminal vesicle and prostate respectively.

AP by *oral* route showed no androgenic activity except for slight elevation of prostate weight at 2-mg dose, an effect which decreased at 4 and disappeared at 8 and 16 mg.

P *orally* produced no stimulation of seminal vesicle and slight (though not dose-related) stimulation of the prostate. At 5 mg, the prostate response was equal to that with 2 mg of ET.

ET *orally* was the most potent prostate stimulator and was exceeded only by ENT and EN in ability to stimulate the seminal vesicle. By *subcutaneous* route it was the most effective stimulator of both seminal vesicle and prostate.

ENT *orally* produced substantial stimulation of seminal vesicle, being about twice as potent as ET; in stimulating the prostate it was one-fourth to one-half as active as ET and approximately equal to P. *Subcutaneously,* it was the second most potent of the compounds tested, being somewhat less than half as potent as ET as a seminal vesicle stimulator and less than one-fourth ET on the prostate.

EN *orally* was the most potent seminal vesicle stimulator of progestins studied, although its dose-response curve was sufficiently different from those of other compounds to prohibit potency estimate. Its action on the prostate was less than that of P or ENT, being only about one-fourth as active as the latter compound. By the *subcutaneous* route it also had a rapidly-plateauing curve in its effect on the seminal vesicle, and had virtually no effect on the prostate at doses used.

MNT was only tested *subcutaneously,* and by this route of administration was inert as seminal vesicle stimulator and produced only minimal stimulation of the prostate.

These results are interesting from the standpoint of differential sensitivity of the 2 glands, supporting previously established concept(6) that seminal vesicle and prostate grow differentially under the influence of compounds of varying chemical structure. ET, for example, when given orally, is more potent than ENT as a prostate-stimulator, but less potent as a stimulus to the seminal vesicle. This shift in relative activity also occurs when one compares EN with ET.

The tendency of EN and of ENT to be more potent in causing weight increase of the seminal vesicle than of the prostate may be associated with "estrogenic" activity of these 2 agents. EN contains a potent estrogen(4), and ENT has been shown(3) to be estrogenic to a significant degree. Ability of estrogen to augment seminal vesicle response to androgen while having little effect on prostate response to androgen has been known for many years (7).

The shifts in activity which accompany varying routes of administration are becoming so commonplace that those observed here scarcely require comment. EN, for example, is more potent in stimulating the seminal vesicle than is ET orally, but is less potent than ET subcutaneously. This is reminiscent of the relative lack of progestational activity of the major component of the EN mixture (norethynodrel) by subcutaneous route compared with its marked activity by oral route in the rabbit(4).

One important fact must be kept in mind in analyzing these results: if the capacity to stimulate male sex accessories is considered indicative of an "undesirable side effect" in clinical situations, the relative androgenic potency of the series of compounds must be considered in relation to the potency of therapeutic activity. The progestational potencies of these compounds vary widely(3,4,5,8,9). Thus, although MAP may be one-eighth as potent a seminal vesicle stimulator as ET by the oral route, it is hundreds of times as potent a progestin(8). Thus for an effective progestational dose of MAP one gives an infinitesimal amount of androgenic potential compared with that of an effective progestational dose of ET.

Summary. When tested for ability to stimulate seminal vesicles and prostates of castrate, immature rats, a series of progestational steroids produced results as follows: 1) None of the compounds has androgenic activity approaching that of standard androgens, methyltestosterone or testosterone propionate. 2) All compounds tested *orally* produced some stimulation of the prostate, although it was extremely slight in the case of 17α-acetoxy progesterone and 6α-methyl-17α-acetoxy progesterone. Significant stimulation of seminal vesicles was seen only with 17α-ethinyltestosterone, 17α-ethinyl-19-nortestosterone, and EN. 3) Of compounds tested *subcutaneously*, only 17α-(2-methallyl)-19-nortestosterone failed to stimulate the seminal vesicle. All compounds produced some stimulation of the prostate, although EN and 17α-(2-methallyl)-19-nortestosterone produced minimal responses.

Samples of Enovid and 17α-(2-methallyl)-19-nortestosterone were generously supplied by Dr. Lee Hershberger and Dr. V. A. Drill respectively, G. D. Searle and Co. All other steroids used were provided by Dept. of Chemistry, The Upjohn Co.

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Particle Counts of Meningopneumonitis Virus by Phase Microscopy.* (24691)

K. O. SMITH AND G. P. MANIRE
Dept. of Bacteriology and Immunology, University of North Carolina School of Medicine, Chapel Hill

A rapid and simple technic for counting psittacosis virus particles would be of obvious value. Gogolak(1) described a modification of the method of Backus and Williams(2) for counting murine pneumonitis virus particles in which virus was mixed with a suspension of known concentration of *E. coli,* sprayed onto a glass slide and stained with Gram stain. The ratio of virus particles to bacteria was determined by counting each by light microscopy. This method was criticized by Crocker(3) on the basis that proof was lacking that the proportions of virus particles and bacteria are unchanged by the staining procedure, and that it does not allow differentiation between virus and non-infectious, non-elementary body forms. The need for a simple and rapid method for counting unpurified and partially purified virus preparations prompted the present study. The technic of a method and confirmation of its validity by egg infectivity tests, electron microscopy counts, and nitrogen determination is reported here.

Materials and methods. (1) *Preparation of virus suspensions.* Meningopneumonitis virus from the American Type Culture Collection was passed serially approximately 20 times in

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the allantoic cavity before use in this study and was known to reach a maximum titer in allantoic fluid 5-6 days after injection. Allantoic fluid suspensions of virus were stored in 3-5 ml volumes at –20°C for use as seed. For use, seed virus was thawed, diluted 1-10 in nutrient broth, and 0.2 ml volumes were injected into the allantoic cavity of 7-day chick embryos. All eggs were incubated for 5-6 days, candled and the dead embryos discarded. The remaining eggs were chilled for 30 min. at –20°C and the allantoic fluids were collected separately. The allantoic fluids were centrifuged at 1,000 rpm for 15 min at 5°C and supernatant fluids were removed by suction and kept at 4°C until titrated. Particle counts and egg titrations were completed within 5 hours after harvest. (2) *Preparation of latex particles*. A polystyrene (latex) particle suspension[†] composed of particles 0.81 micron in diameter was counted in a Petroff-Hausser bacteria counting chamber and diluted in distilled water to contain $10^{9.14}$ particles per ml. The standard suspension was found to be stable when kept at 4°C for up to two months. There was little aggregation of particles during storage, and a particle count at the end of this time was almost identical to that originally obtained. Bacterial growth was not apparent. (3) *Particle counts*. Five-tenths ml amounts of virus suspensions were added to 0.5 ml of the latex particle suspension. After thorough mixing, thin smears approximately 15 mm in diameter were made with a transfer loop on clean slides, air dried and fixed by heat. These smears were stained 5 min with freshly prepared and filtered 0.25% aqueous basic fuchsin, washed gently in tap water for about 3 seconds, dipped briefly into 0.5% citric acid and washed again very briefly in tap water. Dried smears were examined under oil immersion using phase contrast microscopy. A grid was placed inside one ocular to facilitate counting. The number of latex particles and the number of virus particles in several grid areas were determined. No difficulty was encountered in distinguishing between the latex and virus particles. Sufficient grid areas were counted so that no less than 90 of one or the other type particles was counted. The ratio of the virus count: latex count was determined and the virus particle count per ml of allantoic fluid was calculated. (4) *Egg infectivity titration*. Two fold dilutions of infected allantoic fluids were prepared in phosphate buffered saline (PBS) after the virus particle count was determined as above. Four 6-7 day embryos were injected with each dilution *via* yolk sac route. All eggs were candled after 48 hours and dead embryos were discarded. Deaths were recorded for 10 days. Egg LD_{50} per ml was calculated by method of Reed and Muench(4). (5) *Total nitrogen determination*. The method of Lanni, Dillon and Beard(5) was used.

Results. 1. *Precision of method*. Reproducibility of the counts obtained by this method was studied by making 10 separate counts on a single slide prepared as above. The ratios of virus: latex particles ranged from 1.47:1 to 2.0:1 with an average of 1.76:1. The maximum deviation from the average was 16%. Three separate experiments were performed on different days to compare the number of egg infectious units and virus particles per ml of allantoic fluids in individual eggs. The virus counts were made first and the dilutions for egg infectivity tests were based on these counts. These results are shown in Table I. The number of particles per egg LD_{50} varied from 83 to 240 with an average of 145. This is comparable to the findings of Crocker(3).

2. *Effect of washing on the virus:latex particle ratio*. The possibility that particles of either type were washed off the slides during

TABLE I. Relationship of Particle Counts by Phase Microscopy to Egg Infectivity of Meningopneumonitis Virus.

Exp.	Egg No.	Log of virus particles/ml	Log of egg LD_{50}/ml	No. of virus particles per egg LD_{50}
A	1	9.14	7.01	135
	2	9.21	7.29	83
	3	9.01	6.76	178
B	1	9.68	7.30	240
	2	9.57	7.50	118
	3	9.42	7.28	138
C	1	8.81	6.41	174
	2	9.21	7.25	91
	3	8.70	6.43	186

[†] Obtained from Dow Chemical Co.

TABLE II. Effect of Prolonged Washing of Smears on the Virus : Latex Ratio.

Time of washing	Ratio of virus particle : latex particles	Log of virus particles/ml allantoic fluid
5 sec.	1.76	9.39
25	1.80	9.39
2 min.	1.68	9.37
10	1.58	9.34

the staining process was tested by preparing 4 smears from the same virus:latex mixture. All smears were treated the same except for the terminal washing procedure, when the 4 slides were washed for 5 sec, 25 sec, 2 min and 10 min respectively. The virus particle:latex particle ratio was determined on each preparation as above and is shown in Table II. In a second experiment to determine whether washing affected the virus:latex ratio, two smears were prepared from the same preparation, allowed to air dry and were heat fixed. One slide was examined directly and the other was stained before examination. Counts were made on both slides using phase contrast microscopy. Virus particles were plainly visible in the unstained preparations but were counted with more difficulty than in the stained preparation. Triplicate counts were done on each slide. The ratios of virus:latex particles in the unwashed preparation were 0.39, 0.43, and 0.37 (average = 0.40). In the stained preparations the ratios were 0.34, 0.43, and 0.45 (average 0.41). Results from both experiments indicate that no significant loss of particles occurred during the washing process or that both virus and polystyrene particles were washed away at exactly the same rate.

3. *Comparison of counts obtained by phase contrast and electron microscopy.* The morphology of particles counted by the phase contrast technic was studied by electron microscopy.‡ Two suspensions were prepared for counting by sedimenting the allantoic fluid virus at 10,000 rpm for ½ hr, resuspending in distilled water, repeating the process twice more and resuspending to original volume in distilled water. The usual mixture of virus suspension and latex was made and ratios determined by the phase contrast method. Samples from these same virus:latex mixtures were sprayed on collodion membranes and micro-droplets examined with the electron microscope. Latex particles, small-type virus particles, and large-type virus particles were counted separately. The results are shown in Table III. It is seen that the virus counts determined by the phase contrast method average 25% higher than those obtained by electron microscopy. This higher value is probably due to the counting of some virus fragments by the former method. Subsequent similar experiments have demonstrated that this difference is relatively constant. Stained smears of virus:latex mixtures were also found to be plainly visible and readily differentiated by darkfield microscopy. Particle count determinations on the same preparations by phase contrast and darkfield microscopy were found to be almost identical. The use of stained smears was much superior to unstained smears in both cases.

4. *Relationship between particle counts and nitrogen content of washed virus preparations.* In each of 2 experiments, pooled allantoic fluids from infected eggs were centrifuged at 1500 rpm for 30 min to remove cell debris. Supernatant fluids were centrifuged at 10,000 rpm for 30 min in the Spinco #30 rotor. The sediments were resuspended in PBS and washed twice by alternate centrifugation and

TABLE III. Comparison of Particle Counts Made by Phase Contrast and Electron Microscopy.

	Phase microscopy	Electron microscopy		
Exp.	Log virus particles/ml	Log elementary body forms/ml	Log large forms/ml	Log total virus/ml
1	9.32	9.08	8.72	9.24
2	9.27	9.04	8.61	9.18

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‡ The electron micrographs from which these counts were done were obtained through the courtesy of Dr. D. Gordon Sharp.

resuspension prior to nitrogen determination. The results of these experiments are shown in Table IV. A very close correlation between virus counts and nitrogen content is evident, with the average being 9.7 x 10^7 particles per μg of nitrogen.

TABLE IV. Relationship between Nitrogen Content and Virus Particle Counts of Washed Suspensions.

Virus suspension No.	Particles/ml ($\times 10^9$)	μg N/ml	Particles/μg N ($\times 10^7$)
136-1	4.6	47	9.8
-3	6.0	68	8.8
137-1	10.0	97	10.3
-2	10.0	102	9.8
-3	9.1	96	9.5

Discussion. These results have demonstrated that the virus particle counts of meningopneumonitis virus in allantoic fluid obtained by this technic are consistently parallel to egg infectivity titers and nitrogen content. In 9 separate determinations number of particles per egg LD_{50} ranged from 83 to 240 with an average of 145. In 5 separate determinations number of particles per μg of nitrogen ranged from 8.8 x 10^7 to 10.3 x 10^7.

It is certain that numerous non-elementary body forms have been included in the counts done by this technic but the proportion of these to elementary bodies is seldom more than 1:1 as seen by electron micrographs of virus in allantoic fluid and usually the large forms account for about ¼ of the countable particles in electron micrographs. For rapid estimation of virus content of allantoic fluids this technic has been of particular value, and allows the selection of high titer fluids as well as use of a narrow range of dilutions for egg LD_{50} determinations.

The use of latex particles for this procedure has several advantages. This material is stable on storage for long periods of time, is readily available in a variety of particle sizes and is easily standardized by counting in a bacteria counting chamber. The particles are easily visualized by phase contrast microscopy and are readily differentiated from the virus.

Summary. A rapid method for estimating the meningopneumonitis virus content of allantoic fluid suspension is described. Evidence is presented which indicates that counts obtained closely parallel egg infectivity titers and nitrogen content determinations. In 9 separate experiments one egg LD_{50} was found to contain 83-240 virus particles as counted by this method.

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Hepatic and Splenic Uptake of Colloidal Radiogold in Rat after Partial Hepatectomy.* (24692)

KURT STERN AND ARLENE DUWELIUS
Mount Sinai Medical Research Fn., and Depts. of Pathology, Mount Sinai Hospital and Chicago Medical School, Chicago, Ill.

The subject of this study, *viz.*, phagocytic activity of hepatic and splenic macrophages in the rat after partial hepatectomy, has received only scant attention.

Materials and methods. Healthy 2½ to 6 months old Wistar and Lewis rats were used. Twenty-four hours before hepatectomy or sham operation, they were injected intraperitoneally with 0.2 to 0.3 μc/g body weight of radiogold (Abbott's Aurcoloid). Removal of approximately ⅔ of liver was done according

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TABLE I. Hepatic Uptake of Au^{198} in Lewis Rats after Hepatectomy.

Group		Sex	No. of rats	% of inj. Au^{198} per g of liver, mean ± S.D.	P
A	Sham	♂	25	1.79 ± .74	<.001
	Hepatectomy	"	28	3.30 ± 1.22	
	Sham	♀	16	4.26 ± 1.96	>.3
	Hepatectomy	"	22	4.86 ± 2.54	
B	Sham	♂	17	1.98 ± .61	<.001
	Hepatectomy	"	21	3.40 ± 1.14	
	Sham	♀	16	3.59 ± 1.49	<.05 >.01
	Hepatectomy	"	13	4.92 ± 1.38	
C	Sham	♂	11	1.39 ± .68	>.5
	Hepatectomy	"	15	1.26 ± .50	
	Sham	♀	12	2.88 ± 1.42	>.5
	Hepatectomy	"	16	2.72 ± 1.08	
	Liver removed at hepatectomy	♂	65	1.26 ± .99	<.001
		♀	52	3.34 ± 1.82	

A, 3 days after operation; B, 8 days after operation; C, 16 days after operation.

to methods described by Higgins and Anderson(1) and Brues *et al.*(2). Sham operation consisted of laparotomy equivalent to that performed for hepatectomized animals and of brief evulsion of the 2 anterior liver lobes from the incision. Liver samples from hepatectomized animals were weighed, homogenized with distilled water and formamide according to the method by Lahr *et al.*(3) and assayed for radioactivity by liquid counting in Geiger-Mueller counter. A second injection of radiogold of similar dosage was administered 24 hours before anticipated time of sacrifice, which took place 3, 8, 15 or 16 days, respectively, after hepatectomy. Immediately after death of animals, livers and spleens were completely removed, weighed, and converted into tissue homogenates for radioassay. Radioactivity was calculated for whole liver and spleen as well as per g of liver and spleen, with due consideration given to radiodecay of Au^{198}, and expressed as % of injected radiogold. When calculating radioactivity in liver of hepatectomized animals, radioactivity found in liver removed at time of hepatectomy was subtracted from activity of first injection of Au^{198} given to the animal. Mean values and their standard deviations were determined according to usual methods. Tests for statistical significance of differences between values were carried out according to student's t test.

Results. Table I summarizes data on uptake of radiogold/g of liver of Lewis rats. Sham-operated and hepatectomized rats of same sex are compared with each other. Three days after hepatectomy, male animals showed significantly greater uptake in the liver than did sham-operated ones. In female rats, there was only a slight difference in favor of hepatectomized animals 3 days after operation. Eight days following hepatectomy or sham operation, higher uptake in male hepatectomized rats was highly significant, and in female rats of borderline significance. Sixteen days after hepatectomy, male and female rats showed values essentially identical with those found in controls. In all instances, females showed significantly higher hepatic uptakes than did males of comparable groups.

Table II deals with uptake of radiocolloid in spleens of these rats. Three days after operation, in male hepatectomized rats the uptake was about twice as great as in controls whereas in female animals no such difference was found. Eight days after operation, male and female hepatectomized rats showed an increase over controls of borderline significance. Sixteen days after operation, there was no essential difference between hepatectomized and control males, and in female hepatectomized rats the uptake was lower than

TABLE II. Splenic Uptake of Au^{198} in Lewis Rats after Hepatectomy.

Group		Sex	No. of rats	% of inj. Au^{198} per g of spleen, mean ± S.D.	P
A	Sham	♂	25	1.89 ± 1.36	<.001
	Hepatectomy	"	28	3.90 ± 1.72	
	Sham	♀	15	4.01 ± 1.70	>.5
	Hepatectomy	"	22	4.22 ± 1.97	
B	Sham	♂	17	2.23 ± 1.25	<.1 >.05
	Hepatectomy	"	21	2.92 ± 1.02	
	Sham	♀	15	2.74 ± 1.30	<.1 >.05
	Hepatectomy	"	13	3.70 ± 1.60	
C	Sham	♂	11	2.82 ± 1.69	>.1
	Hepatectomy	"	15	2.00 ± .87	
	Sham	♀	12	4.06 ± 2.34	>.1
	Hepatectomy	"	15	2.91 ± 1.40	

A, 3 days after operation; B, 8 days after operation; C, 16 days after operation.

TABLE III. Hepatic Uptake of Au^{198} in Male Wistar Rats after Hepatectomy.

Group	No. of rats	% of inj. Au^{198} per g of liver, mean ± S.D.	P
A Sham	24	1.51 ± .84	<.001
Hepatectomy	23	2.92 ± 1.44	
B Sham	17	1.71 ± .76	>.3
Hepatectomy	18	1.97 ± .79	
Liver removed at hepatectomy	41	1.04 ± .71	

A, 3 days after operation; B, 15 days after operation.

in controls, though the difference was not statistically significant. In the spleen, too, uptake of Au^{198} was greater in females than in males of corresponding groups.

In male Wistar rats phagocytic activity was estimated 3 and 15 days, respectively, after hepatectomy. As shown in Table III, uptake of radiogold in liver was markedly increased 3 days after hepatectomy, whereas 15 days later there was no significant difference. Table IV shows Au^{198} uptake in spleen of hepatectomized Wistar rats was significantly increased 3 days and 15 days after hepatectomy.

Since uptake of Au^{198} was also known for hepatectomy specimens these values were compared with those obtained at time of sacrifice. In Table V, the last vertical column refers to number of rats in which at sacrifice Au^{198} uptake/g of liver was at least twice as great as in the hepatectomy specimen. Two-thirds of male Lewis rats showed ratios over 2, three days after hepatectomy, and three-fourths of them 8 days after hepatectomy, but only 1 of 15 rats, 16 days after hepatectomy. About ½ or ⅓ respectively of female Lewis rats showed increased uptake 3 and 8 days after hepatectomy, and none 16 days after hepatectomy. Of male Wistar rats, roughly ⅔ showed increased uptake 3 days after hepatectomy, and ½ at 15 days.

TABLE IV. Splenic Uptake of Au^{198} in Male Wistar Rats after Hepatectomy.

Group	No. of rats	% of inj. Au^{198} per g of spleen, mean ± S.D.	P
A Sham	24	1.77 ± .99	<.001
Hepatectomy	22	2.44 ± .83	
B Sham	17	1.55 ± .86	<.01
Hepatectomy	18	1.95 ± 1.01	

A, 3 days after operation; B, 15 days after operation.

An additional effect of partial hepatectomy was noted in weights of spleens. Comparison of splenic weights expressed as % of body weight disclosed in male and female hepatectomized Lewis rats 8 days after hepatectomy a significant increase in relative size of spleen as compared with controls, and an increase in hepatectomized Wistar rats, 3 and 15 days after hepatectomy of borderline significance.

TABLE V. Comparison of Au^{198} Hepatic Uptake Determined at Hepatectomy and at Sacrifice.

Strain	Sex	Days after hepatectomy	No. of rats Total	$\frac{S}{H} > 2$*
Lewis	♂	3	28	18
	♀	3	22	10
	♂	8	20	16
	♀	8	13	4
	♂	16	15	1
	♀	16	16	0
Wistar	♂	3	23	16
	”	15	18	10

* $\frac{\text{Uptake at sacrifice}}{\text{Uptake at hepatectomy}} > 2.$

Discussion. Our data suggest that after partial hepatectomy of rats, liver and spleen are endowed with increased phagocytic activity. This may be the result of proliferation of hepatic and splenic macrophages, an assumption supported by increase observed in relative splenic size and preliminary histologic studies. While female rats showed greater phagocytic activity than males, the effect of hepatectomy was less pronounced in female than in male rats, possibly because it is more difficult to stimulate phagocytosis operating near maximum efficiency.

Benacerraf and co-workers(4) observed that partial hepatectomy and other measures reducing portal blood flow in rats, interfered with blood clearance of intravenously injected india ink, but enhanced hepatic phagocytosis, presumably because of increased blood flow through remaining portion of liver. It is conceivable that increased blood flow/volume of tissue following partial hepatectomy may have contributed to the increase of phagocytosis also in our experiments, but since we measured phagocytosis 24 hours after intraperitoneal injection, it is less likely that this was the decisive factor. Simultaneously with preliminary report of our results(5), Leong and associates(6) presented data obtained by isolated rat liver preparations perfused with radioactive colloidal chromic phosphate. In this study the authors found that 48 hours after hepatectomy phagocytic activity of liver had doubled over that of controls. Since these results were obtained with controlled rates of perfusion *in vitro,* they do not support the assumption that increased rate of blood flow was the main factor in elevating hepatic phagocytosis after partial hepatectomy. Abercrombie and Harkness(7) observed that regeneration of littoral cells proceeded at a faster rate than that of parenchymal cells between 2 and 7 days after partial hepatectomy. Similarly, Aterman(8) noted an increase in sinusoidal cells as early as 3 hours after partial hepatectomy It is also of interest to refer to observations by Haven *et al.*(9) on increased immune responses in rats after partial hepatectomy; when injections of sheep red cells were given immediately after hepatectomy, these animals showed significantly higher hemolysin titers than did controls.

Summary. 1. Uptake of Au^{198} in liver and spleen was compared after hepatectomy or sham operation was carried out in Lewis and Wistar rats. 2. Female rats showed higher uptake of radiogold in liver and spleen than did males. 3. Three and 8 days after hepatectomy, male Lewis rats, and 3 days after hepatectomy male Wistar rats, showed greater uptake of radiogold in liver and spleen than did sham-operated controls. Similar but less pronounced differences were found in female Lewis rats. 4. Fifteen or 16 days after hepatectomy male and female Lewis rats showed no significant differences in hepatic or splenic uptake of radiocolloid, while in male Wistar rats splenic but not hepatic uptake was still increased. 5. Increased spleen/body weight ratios were observed in hepatectomized rats.

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The Free Myo-Inositol of Thyroid Tissue.* (24693)

NORBERT FREINKEL, REX M. C. DAWSON, SIDNEY H. INGBAR AND ROBERT W. WHITE
Thorndike Memorial Lab., Second and Fourth Medical Services (Harvard), Boston City Hospital, and Dept. of Medicine, Harvard Medical School, Howard Hughes Medical Inst., and Biochemistry Dept., A. R. C. Inst. of Animal Physiology, Babraham, Cambridge, England

During a study of phospholipid metabolism of sheep thyroid tissue(1-3), abundant quantities of a water-soluble compound were noted which exhibited chromatographic mobility and staining characteristics of free myo-inositol. In addition, in lipid extracts of sheep thyroid tissue, an inositol-containing phosphoglyceride was demonstrated. *In vitro*(2), as well as *in vivo*(3), the P^{32} turnover in this phosphoinositide was 2 to 10 times more rapid

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than that of any other lipid component. Comparable earlier observations are limited. In 1946, Meyer, on the basis of C and H analysis and melting-point determinations, identified as inositol the crystals which appeared in alcoholic extract of commercial desiccated thyroid powder(4). He estimated that the "inositol" accounted for 0.3% of the powder and cited Tambach's original report in 1896 of "the presence of inositol in aqueous extracts obtained from thyroids in manufacturing process"(5). The present studies were designed to provide more definitive information about the thyroid content of inositol. Levels of free myo-inositol in the thyroid gland of a variety of species have been determined by specific microbiological assay(6). Preparation and extraction of tissues were designed to minimize the breakdown of inositol-containing phospholipids, proteins, and water-soluble phosphate esters.

Methods. Specimens of human thyroid were obtained at surgery of patients with Graves' Disease or non-toxic nodular goiter. Sheep thyroid glands were obtained immediately following exsanguination of animals at abattoir. Rabbit, rat, and guinea pig glands were rapidly removed from animals which had been anesthetized with ether or stunned by blow on head. The excised tissues were placed directly on powdered solid CO_2 for quick-freezing and then extracted by homogenization with iced 5% perchloric acid (PCA) in Potter-Elvehjem ground-glass homogenizers. The chilled acid extracts were separated from insoluble residues by centrifugation and filtration through Whatman #42 filter paper. PCA in aliquots of supernatant was precipitated by neutralization with 5 N KOH at 0°C. Whenever possible, specimens of heparinized blood were obtained simultaneously and aqueous extracts of blood and plasma were prepared by precipitating plasma proteins and lipids with PCA. Neutralized filtrates were diluted so that 1 to 5 μg inositol were contained in 2.5 ml. This volume was combined with equal quantity of medium described by Campling and Nixon(6) and, after autoclaving, was inoculated with Kloeckera Brevis yeast and grown for 3 days at 25°C. Yeast growth was assessed by turbidimetric assay. All samples were analyzed in triplicate and related to duplicate standard curves which were obtained concurrently.

TABLE I. Concentration of Free Myo-Inositol in Thyroid Glands and Plasma of Various Species.

	Free myo-inositol*					
Species	Thyroid (mg/100 g)†			Plasma (mg/100 ml)		
Human	126	76–166	(4)	.45	.43– .46	(4)
Rabbit	200	126–350	(4)	.91	.62–1.20	(2)
Rat	135	56–255	(12)	1.22	.90–1.61	(5)
Guinea pig	372	215–550	(8)	1.70	.92–3.67	(4)
Sheep	355	276–432	(4)			

* Values represent mean and range. No. in parentheses represent No. of individuals in each group.

† Values derived on basis of wet wt of fresh thyroid tissue.

Results are listed in Table I. The Kloeckera Brevis yeast assay measures only free myo-inositol. By this criterion, the mean thyroid/plasma concentration differentials for free myo-inositol were greater than 100 in every species examined. Hydrolysis of aqueous extracts in 5 N HCl for 20 hours at 105°C did not increase inositol concentration thus indicating that the thyroid content of inositol phosphates is inappreciable. Enhancement of "free myo-inositol" due to liberation of inositol by hydrolysis of inositol esters during preparative manipulations does not seem likely. Similar concentrations of free myo-inositol were found in control studies with guinea pig thyroid wherein a) one lobe was excised immediately and frozen on solid CO_2 (mean 445 mg/100 g) and b) the second lobe was left i*n situ* for 4 minutes prior to excision (mean 465 mg/100 g). Moreover, levels of free myo-inositol were not significantly different in thyroids of anesthetized and "stunned" animals thus precluding the likelihood of an anesthesia artifact. Finally, paper chromatography of aqueous extracts of guinea pig thyroid in ethanol/NH_3, isopropanol/acetic acid, and cellosolve/butanol/ethanol/NH_3 solvents confirmed the presence of considerable amounts of inositol in the gland.

Discussion. The thyroid gland of every species examined contained abundant quantities of free myo-inositol. On the basis of known compositional characteristics of sheep thyroid tissue, some quantitative dimensions may be assigned to the free inositol in this species. Since average dry weight of sheep thyroid gland constitutes 20.3 ± 3% of wet weight(7), the average inositol level of 355 mg/100 g observed in the present study would account for 1.7% of dry weight of ovine thyroid. In terms of ultimate disposition, only a small fraction of this free inositol need be necessary for incorporation into phosphoinositide. For example, lipid phosphorus in the sheep thyroid averages 191.5 μg P/g wet weight and 9% of this is present in the phosphoinositide(2). Thus, assuming that thyroid phosphoinositide is a monophosphoinositide, its inositol content maximally would equal only about 0.3% of free inositol.

For the moment, the physiological significance of free myo-inositol in thyroid tissue is unclear, nor has it been determined whether the thyroid can concentrate or synthesize inositol. Eagle has demonstrated the role of myo-inositol as an essential growth factor in tissue culture(8). Tissue analyses in rats, guinea pigs and rabbits (to be published elsewhere), have indicated that free myo-inositol is significantly more abundant in thyroids of these species than in salivary gland, heart, liver, spleen, kidney cortex, adrenal, pancreas, testicle, ovary, lung, mammary gland and diaphragm. Screening studies of multiple tissues have indicated some correlation between levels of free inositol and protein elaboration. Thus, conceivably, the abundant myo-inositol within the thyroid could be linked to the active intrathyroidal accumulation of amino acids (or other anions such as iodide), protein synthesis, or secretion of thyronines. Alternatively, in view of known conversion of inositol to glucuronic acid(9,10) some of the free myo-inositol within the thyroid might be implicated in active formation of intrathyroidal mucopolysaccharides(11). Experimental efforts to assess these and other possibilities and to correlate inositol levels with thyroid activity are in progress.

Summary. Bioassay with Kloeckera Brevis yeast has been employed to measure content of free myo-inositol in human, rat, rabbit, sheep and guinea pig thyroid glands. In all species, thyroid/plasma concentration differentials for free myo-inositol were greater than 100. Chromatographic documentation has also been secured, and the possible significance of the abundant intrathyroidal myo-inositol has been discussed.

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Propagation of Canine Distemper (CD) Virus in Tissue Culture. (24694)

V. J. Cabasso, K. Kiser and M. R. Stebbins (Introduced by H. R. Cox)
Viral and Rickettsial Research Section, Lederle Labs., American Cyanamid Co., Pearl River, N. Y.

Repeated attempts by the authors to grow CD virus (canine distemper virus) of dog or ferret origin in tissues of those 2 species yielded essentially negative results. Presumably experience of others has been similar; no report of a successful attempt has come to our attention, among numerous publications in the past 5 years on tissue-culture propagation of many different animal viruses. This communication describes the successful propagation in chick-embryo tissue culture of chick-embryo-adapted CD virus, with no evidence of cytopathogenic effect.*

Materials and methods. Virus strains. American Cyanamid (Lederle) strain of chick-embryo-adapted CD virus(1) was used. Experimental ferrets were challenged with American Cyanamid (Lederle) stock strain of virulent CD virus(2). *Tissue culture.* The virus was serially passed in stationary tube cultures of trypsinized total chick-embryo tissues (9 - day - old embryos) prepared essentially by the method of Dulbecco and Vogt(3). Povitsky bottle cultures of same tissue were used for virus pools for dried experimental vaccine. Growth medium for both cultures was Eagle's solution (4) containing 10% horse serum, in amounts of 1 ml/tube and 100 ml/Povitsky bottle. After 24-hour growth at 37°C, cultures were renewed with mixture 199(5), and thereafter no changes were made. *Virus titrations.* Live 7- or 8-day-old chick embryos were inoculated on the chorioallantoic membrane (CAM) by the technic described by Gorham (6). Six embryos were inoculated with 0.2 ml doses of each serial 10-fold dilution of infected tissue-culture fluid. After 7 days incubation at 35°-36°C, results were recorded by removing the top of each CAM and examining it under a strong light for lesions. Infected membranes were thickened and showed the opaque foci associated with American Cyanamid (Lederle) strain of CD virus. Fifty-%-chick-embryo-infective end points (CED_{50}) were calculated by the Reed and Muench formula (7). *CD dog immune serum.* To prepare serum, a susceptible puppy was injected subcutaneously with 2 ml of a 30% CD-infected CAM suspension. Repeated titrations of this serum by the neutralization method gave values between $10^{-3.0}$ and $10^{-4.0}$ against virus CED_{50}'s of 20 to 200. Before use in the neutralization test, both immune and normal sera were inactivated at 56°C for 30 minutes. *Neutralization test.* Triple sets of 10-fold dilutions of infected tissue-culture fluids were prepared in hormone broth. Dilutions of the first set were mixed with equal volume of hormone broth, the second set with equal volume of 1:8 dilution of CD immune dog serum, the third set with equal volume of 1:8 dilution of normal dog serum. After standing at 4°C for 4 hours, each mixture was inoculated in doses of 0.2 ml on the CAM of 6 chick embryos, as described above. After 7 days incubation at 35°-36°C, results were recorded and 50% endpoints calculated, as also described above. *Susceptible ferrets.* These were procured and maintained under the same conditions of strict isolation reported previously(2).

Results. Serial propagation of CD virus in tube cultures. The 48th passage of CD virus in live embryos was inoculated into 10 chick-embryo-culture tubes, 0.1 ml of a 40%-infected CAM suspension/tube. Tubes were incubated at 37°C and examined microscopically for 7 days. No gross cytopathogenic effect was observed. Monolayers then were detached into the fluid, pooled, and ground in Tenbroeck grinder. The undiluted suspension was inoculated on the CAM of chick embryos, 0.2 ml/egg. A second tissue-culture passage then was made in 10 new tubes, 0.2 ml of suspension/tube. Again virus was detected in the inoculated embryos, but no cytopathic changes occurred in tissue-culture passage. Serial transfers were continued in the same manner every 7 days for 14 passages. None showed cytopathogenicity, but in undiluted form all produced on the CAM lesions characteristic of CD virus.

From 15th passage on, in total of 64 passages, all without cytopathology, tissue-culture harvests were not inoculated as undiluted

TABLE I. Optimum Time of Harvest of CD Virus Propagated in Chick-Embryo-Tissue Culture.

Day of harvest	CED_{50}/ml Passage level 19th	 20th
4	2.9*	4.2
5	1.9	3.4
6	2.7	2.4
7	2.4	3.2

* Power of 10.

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* The following article, on cultivation of a "street" strain of CD virus in dog kidney tissue culture, came to the attention of the authors of this manuscript after it had been submitted for publication. Rockborn, G. Canine Distemper Virus in Tissue Culture, *Archiv Virusforsch.*, 1958, v8, 485.

TABLE II. Serum Neutralization Results of Tissue-Culture-Grown CD Virus, and 1st Chick-Embryo Passage Thereof.

Virus suspension plus:	Passage level: CED_{50}/ml					
	Tissue culture			Tissue culture plus 1 chick-embryo passage		
	21st	34th	55th	21st	34th	55th
Sterile broth	3.4*	3	3	4	4	4
Normal dog serum	3	3.5	3	4	3.5	3.7
CD-immune dog serum	<1	<1	<1	<1	<1	<1

* Power of 10.

suspensions but, instead, were titrated in the chick embryo. Titers ranged from $10^{2.1}$ to $10^{5.2}$/ml, the majority falling between $10^{3.0}$ and $10^{4.5}$. These values are of the same order as those obtained with CAM suspensions of infected live embryos.

To determine optimum time of harvest, tissue cultures of 19th and 20th tissue-culture passages were titrated after 4, 5, 6 and 7 days of incubation. Since results (Table I) indicated that maximum virus yields are reached in 4 days, subsequent passages were harvested routinely on 4th day.

The tissue-cultured virus was identified by the neutralization method and by ferret immunization.

Serum neutralization. Results of this test (Table II), performed at 3 different transfer levels of the virus, in tissue culture and on CAM suspensions derived from their first back-passages in live chick embryos, prove unequivocally the immunological identity of the tissue-culture-propagated agent with the original chick-embryo-adapted CD virus.

Ferret immunization. Two normal ferrets were each inoculated intraperitoneally with 1 ml of undiluted suspensions of 22nd and 34th tissue-culture passages. These animals remained normal for 3 weeks and then they and 2 normal control ferrets were challenged, also intraperitoneally, with virulent distemper virus.

Later, the 42nd tissue-culture passage was diluted in 10-fold steps and 1 ml of each dilution, from undiluted through 10^{-5}, was inoculated intraperitoneally into 3 normal ferrets. During the next 3 weeks 2 ferrets (10^{-2} and 10^{-4} groups) died of pneumonia. The others remained normal, and, together with 2 normal controls, were challenged with virulent CD virus. Results of both experiments (Table III), recorded 26 days after challenge, also established the identity of the tissue-culture agent with CD virus. Control animals died of typical distemper, but all others survived, whether they had been injected with 150,000 or as few as 1.5 CED_{50}'s.

Experimental vaccines. Three experimental lots of vaccine were prepared, 2 from 33rd and one from 34th serial passage of the virus. Povitsky-bottle cultures were inoculated with 10 ml of infected tissue-culture suspensions representing these passages, and harvested 4 days later. Harvests were ground in Waring blendor for 2 minutes, distributed in 2 ml amounts in glass vials, and dried from the frozen state. Virus titrations made on fresh harvests and after lyophilization indicated an average loss of about 1 to 1.5 logs as a result of drying (Table IV). Nevertheless, all 3 preparations were shown in ferret immunization tests to be effective vaccines.

TABLE III. Immunization of Ferrets with Tissue Culture CD Virus.

Exp. No.	Passage level	Inoculum dilution (1 ml)	CED_{50} inj.	Challenge results No. dead from challenge/total No. challenged Immunized ferrets	Control ferrets
1	22	Undil.	2.9*	0/2	2/2
	34	"	3.4	0/2	
2	42	"	5.2	1/2†‡	
		10^{-1}	4.2	0/3	
		10^{-2}	3.2	0/2‡	"
		10^{-3}	2.2	0/3	
		10^{-4}	1.2	0/2‡	
		10^{-5}	.2	0/3	

* Power of 10.

† One ferret died of pneumonia 10 days after challenge; no distemper symptoms.

‡ Immunized ferrets which died of inter-current infection before challenge and showed no distemper symptoms were omitted from Table.

TABLE IV. Titers of Fresh and Post-Drying Experimental Vaccines Prepared in Chick-Embryo Tissue Culture.

Vaccine lot No.	Passage level	CED_{50}/ml Before drying	After drying
13A	33	3.7*	2.7
14A	33	3.7	2.2
15A	34	4.2	3.2

* Power of 10.

Lots 13A and 14A were reconstituted to volume with sterile distilled water and, without further dilution, each was injected intraperitoneally into 3 normal ferrets in 1 ml doses containing, respectively, 500 or 150 CED_{50}'s. Lot 15A, in addition to similar administration to 6 ferrets in undiluted form, also was injected into 3 ferrets each as a 10^{-1}, 10^{-2} and 10^{-3} dilution, in 1 ml doses containing from 1,500 to 1.5 virus CED_{50}'s. Table V shows that, while normal challenge control animals died of the experimental disease, all immunized ferrets survived, regardless of number of CED_{50}'s of tissue-culture-propagated virus injected.

Discussion. Despite continued failure to grow CD virus of dog or ferret origin in cultures of tissues from these species, a chick-embryo-adapted CD virus was propagated successfully in chick-embryo-tissue culture for 64 serial transfers. The observation that gross cytopathology did not accompany virus multipication is not unique. Possibly histological studies, including use of the fluorescent antibody technic, would reveal changes indicative of cellular infection. Such studies are under consideration.

TABLE V. Immunization of Ferrets with Experimental Tissue Culture CD Vaccines.

Vaccine lot No.	Passage level	Inoculum dilution (1 ml)	CED_{50} inj.	Challenge results No. dead from challenge/total No. challenged Immunized ferrets	Control ferrets
13A	33	Undil.	2.7*	0/3	2/2
14A	33	"	2.2	"	"
15A	34	"	3.2	1/6†	4/4
		10^{-1}	2.2	0/3	
		10^{-2}	1.2	"	
		10^{-3}	.2	"	

* Power of 10.

† One ferret died of inter-current infection; no distemper symptoms,

The tissue-culture-grown virus remained antigenic for the ferret through at least 42 passages, at remarkably low virus CED_{50} (0.15 - Table III). This observation suggests that while the chick-embryo CAM is of great practical value, it is less sensitive than the ferret for detection of minute amounts of CD virus, and that, as presently used, the CAM fails to reveal the entire virus content of infected preparations.

Summary. A chick-embryo-adapted canine distemper virus was propagated successfully for 64 passages in cultures of total chick-embryo tissues. As measured on the chorioallantoic membrane of live chick embryos, average virus titers of these passages ranged between $10^{3.0}$ and $10^{4.5}$/ml infected culture suspension. These titers are of the same order as those obtained with live-chick-embryo-grown virus. Identity of tissue-culture-grown agent with original CD virus employed was established by virus neutralization method and by ferret immunization. Effective freeze-dried vaccines were produced with different passage levels of tissue-culture-grown virus.

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Action of Some Sympatholytic Agents on Pregnancy in the Rat. (24695)

F. Bovet-Nitti and D. Bovet (Introduced by M. C. Shelesnyak)
Laboratorio di Chimica Terapeutica, Instituto Superiore di Sanità, Rome, Italy

Shelesnyak(1-3) showed that ergot alkaloids, especially ergokryptine and ergocornine terminated early (pre-implantation) pregnancy and inhibited formation of deciduomata in rats. That these effects were overcome by progesterone led the author to believe that they appear to be exerted on hormonal balance (estrogen-progesterone)(2,3). Chambon(4) demonstrated that chlorpromazine induced abortion in pregnant rat, and similar action by reserpine has been reported (5). Abortive action of both drugs could be prevented by progesterone treatment. Many clinical observations led Cranston(6) to examine action of tranquilizing drugs on normal estrus rhythm of mice. For pharmacologists these hitherto unknown aspects of action of some antagonists of neurovegetative nervous system mediators have great interest because 1) they show a new area of action of these drugs and 2) make evident their particular toxicity in this field. There are, however, very few systematic researches in the field of drugs which act on the neurovegetative nervous system associated with reproductive functions. It is from the standpoint of their pharmacological relationship that we carried out these experiments to examine the action exerted by them on fertility and fecundity of the female rat. Seventeen compounds were tested, many known sympatholytic drugs, and others belonging to other series formerly described by us(7-11). Compounds selected were: 1) for sympatholytic effects, a tetra hydro-β-naphtylamine derivative, N-(2-tetralyl) N-methyl-N′ethyl-β-alaninamide (916IS)(7,8); piperoxan hydrochloride (933F) and 3 of its derivatives, N-(2-methyl - 1,4-benzodioxan)-N′ methyl-β-alaninamide (1205IS)(9). 2) for hypotensive properties, N - piperidine (2-aminomethyl - 1, 4 - benzodioxan) acetamide (1659IS) and 3) for central effects N-morpholine-2-aminomethyl 1,4-benzodioxan-acetamide (1656IS)(10,11).

Methods. In Group I (Table I) females in proestrus were mated with males whose fertility had been established. Presence of sperm in vaginal smear on the next morning was considered evidence of impregnation and onset of pregnancy. On 4th day of pregnancy (5th after coitus) intramuscular injections were given. In Group II (Table II) females were selected as in Group I, but drugs were administered orally for a period of 7 days beginning on the 3rd day after coitus. The animals were in individual cages with especially designed drinking vessels which contained the drugs and which were weighed daily to ascertain the water intake. In Group III, females with established regularity of the sex cycle as determined by vaginal smears, were used to study the action of some drugs on the cycle periodicity. Drugs were administered either intramuscularly or orally, and smears were examined daily during treatment and for 10 subsequent days. Comparison of the number of estrus cycles during 10 post-treatment days with the control number formed the basis for judging the effectiveness of the drug.

Results. In summary the effects of a single intramuscular injected on the 5th *post coitum* (Table I) are: 1) Amphetamine and yohimbine failed to alter the course of pregnancy or litter size. 2) Infundin and pyrilamin acted on the fetus, so that at normal parturition malformed or dead fetuses were expelled, or those which were viable died, soon after birth. 3) Ergotoxin interrupted early pregnancy with the appearance of estrus smears within 72 hr after injection as demonstrated by Shelesnyak (1,2). 4) Quinine caused generalized intoxication. Large doses took rapid effect on early pregnancy—and were fatal to mother; lower doses caused foetal damage. 5) Chlorpromazine and reserpine disturbed the late stages of pregnancy as reported by Chambon(4) and Tuchmann-Duplessis *et al.*(5). 6) With some synthetic derivatives of tetrahydro-β-naphtylamine (621IS; 916IS) and 2-aminomethylbenzodioxan series (1205IS; 1656IS; 1659IS).

TABLE I. Effect of Single Muscular Injection of Some Drugs on Early Pregnancy in Rat.

	Dose, i.m. (mg/kg)	Normal	Aborted*	Lethal effects†‡		Gestations terminated§
				On mother	On litter	
A Amphetamine	10	6/8		2/8		
Yohimbine	20	8/8				
Infundin	1 U.	4/8			3/8	1/8
Pyrilamine	50	3/8			4/8	”
Thonzylamine	”	6/8				2/8
Strychnine	0.5	6/8			1/8	1/8
Chloropromazine	20	4/8			2/8	2/8
Reserpin	0.5	10/16		1/16	2/16	3/16
B Quinine	10	7/16	3/16		1/16	5/16
	100	5/8		1/8		2/8
	250	4/8		2/8	1/8	1/8
C Ergotoxine	20	1/8				7/8
D THN	20	5/8			1/8	2/8
621 I.S.	100	7/16	1/16	1/16	4/16	3/16
916 I.S.	”	6/16	4/16	2/16	1/16	”
E Piperoxan	250	9/12		1/12		2/12
1205 I.S.	100	3/8	1/8	2/8		2/8
1656 I.S.	20	5/8			2/8	1/8
	100	9/16			2/16	5/16
	250	5/16			2/16	9/16
1659 I.S.	100	6/8				2/8
Controls		7.2/8 (avg of 200 rats)				

* *Aborted:* Expulsion of non-viable fetuses between day 12 and 17 of pregnancy.

† *Lethal effects on mother:* Maternal death during course of fetal abortion; or maternal death at term accompanied with massive uterine haemorrhage, prolapse, or retention of litter.

‡ *Lethal effects on litter:* Entire litter dead at parturition or within 24 hr thereafter.

§ *Gestations terminated:* Assignable to failure to implant, degeneration of blastocyst, or hormonal disturbance.

various degrees of disturbance of pregnancy were observed which were partly associated with toxicity related with oxytocic properties.

Results from Group II (Table II) revealed that reserpine was equally effective by injection and mouth, and that 100-160 mg/k/d of 933F, and 150-265 mg/k/d of 1656IS terminated pregnancy in 6/10 to 9/10 of the groups.

From results in Group III it appears that 933F maintains estrus while 1656IS diestrus.

Conclusions. These experiments permitted screening of various drugs, affecting the neurovegetative nervous system, for their action on

TABLE II. Effect of Oral Administration of Drugs during Early Pregnancy in Rat.

	Daily oral dose			Toxic effects		
	Conc. in drinking water, mg/100 cc	Avg dose absorbed (7 days), mg/kg/day	Normal	On mother	On litter	Gestations terminated
Reserpin	2.5	2.5	1/10		3/10	6/10
Piperoxan	200	105	4/10			”
	500	100	1/10		1/10	8/10
	”	164	0/10	1/10*		9/10
1656 I.S.	200	104	7/10		”	2/10
	500	266	1/10			9/10
	”	148	2/10		”	7/10
	”	175	”			8/10
Controls			9/10 (avg of 30 rats)			

* Died on 10th day of pregnancy—undetermined cause.

early and middle pregnancy in the rat. Where effects are noted, they were, mainly, in that pregnancy did not proceed normally. In some instances there was evidence of abortion, in others of toxic action on the mother, or on the foetuses (with resulting death or malformation) or recurrence of estrus (*e.g.*, ergotoxine) or simple persistence of diestrus. No effort was made in these preliminary experiments to pin-point the site or mechanism of action on the course of pregnancy.

The findings of other workers(1-4) were confirmed. Rats treated with 2 drugs of our series, 1656IS and 933F, revealed significant percent of failure to achieve successful pregnancy, but the mechanism of action remains to be elicited.

It appears that pharmacologically active substances may have significantly selective effects on the sexual cycle and on early pregnancy. This is an area of investigation which until now has remained mainly in the domain of endocrinology.

Summary. Drugs acting on the neurovegetative nervous system, particularly certain derivatives (sympatholytic) of the β-tetra hydronaphthylamine and 2-aminomethylbenzodioxan series, were tested for their action on gestation in the rat. Certain agents showed interference with normal pregnancy, but the mechanism of action remains to be explored.

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Effect of Endotoxin on Gastrointestinal Mucosa of the Rat.* (24696)

Selwyn A. Broitman, Alyce L. Bezman, Margaret M. Hazel
and Norman Zamcheck (Introducd by E. H. Kass)
Mallory Inst. of Pathology and Gastro-Intestinal Clinic, Boston City Hospital and
Dept. of Pathology, Boston University School of Medicine

Pathogenesis of the gastrointestinal congestion and hemorrhage caused by endotoxin poisoning(1,2) is not clearly understood. The present investigation was undertaken to study alterations in morphology and biochemical activity of the gastrointestinal tract of the rat during acute endotoxin intoxication. Measurements of succinoxidase activity, an enzyme abundantly distributed in the normal rat gastrointestinal mucosa(3) were used as an indication of aerobic metabolism. An inhibitor of the succinoxidase system in the rat intestinal mucosa has been described by Nakamura *et al.*(4). It was necessary, therefore, to ascertain whether any changes in activity were due to alterations of the inhibitor rather than to changes in enzyme activity *per se*. In addition, parallel studies were carried out to determine if the mucosal barrier to microorganisms was impaired by endotoxin. Invasion by intestinal microorganisms resulting from chemical irritation of intestinal mucosa and other types of gastrointestinal trauma has been observed(5,6) while transmural migration of microorganisms, or products thereof,

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from intestines of experimental animals subjected to hemorrhagic shock has been suggested by Fine and co-workers(7). The failure to culture microorganisms from tissues of rats under the latter condition has been attributed to host antibacterial defense mechanisms. In this study maximal lethal doses of endotoxin were used to minimize host antibacterial defense mechanisms by (1) diminishing efficacy of the reticulo-endothelial system(8,9) and (2) inhibiting diapedesis(1).

Methods. Salmonella typhi endotoxin was prepared according to the method of Levitin *et al.*(10). One ml (6.5 MLD_{50}) was injected into the hearts of ether-anesthetized rats[†] weighing 150-200 g. This dose produced death within 2 to 4 hours. The small intestine of animals sacrificed after varying intervals was immediately removed, flushed and fixed with 10% buffered formaldehyde. Selected tissues were sectioned and stained with hematoxylin and eosin. The small intestines of animals treated comparably were washed with ice cold saline and a homogenate was prepared by scraping the mucosal surface of the entire small intestine with a razor blade and homogenizing for exactly 90 sec. in 10 ml of water. Succinoxidase activity was measured manometrically in triplicate according to the method of Potter and Schneider(11). The micro Kjeldahl method was used for nitrogen determination. Activity of the succinoxidase inhibitor was measured according to the method of Nakamura *et al.*(4), which consisted of determining the suppression of rat heart succinoxidase activity by addition of rat gut homogenate. Routine cultural technics were used in bacteriological studies.

Results. Morphologic changes. After endotoxin injection, the rats exhibited generalized weakness, ataxia, and respiratory distress typical of endotoxin poisoning. Under pentothal anesthesia 6 rats were injected by intracardiac route with endotoxin, laparotomy was performed and visceral changes observed. Onset of edema and congestion of small intestine varied from 20 to 80 minutes after endotoxin injection. This variability was further corroborated by observation in sacrificed rats that 20 of 40 animals had edema and congestion within 60 minutes after endotoxin injection. Benzidene tests for blood in the stool were positive in 6 of 9 rats within 40 minutes after endotoxin injection; all had positive stools at the end of 100 minutes. Grossly bloody stools were not seen nor was diarrhea a constant finding. Fourteen of 16 rats autopsied immediately after death showed marked edema and congestion of small and large intestine and dilatation and engorgement of mesenteric blood vessels. Microscopic examination[‡] revealed congestion and hemorrhage into the lamina propria with disruption of glandular architecture. Migration of red blood cells into the interepithelial spaces and, in some instances, escape of red blood cells into the lumen of intestine were also visualized.

TABLE I. Effect of Rat Serum on Succinoxidase Activity of Normal Rat Intestinal Mucosa.

Animal #	Qo_2/N	Animal #	Qo_2/N
1	202	8	244
2	303	9	150
3	267	10	211
4	256	11	401
5	389	12	103
6	276	13	395
7	154	14	305
		Mean ± S.D. =	261 ± 93

Effect of endotoxin on succinoxidase activity. Previous studies demonstrated that succinoxidase activity of the normal rat intestinal mucosa is exceedingly variable ranging from zero to 400 microliter O_2/hr/mg N. Similar difficulties were encountered by Davenport *et al.*(12) in the mouse stomach. When 0.3 ml of rat serum was substituted for distilled water in Warburg vessels, less variability in respiration (Table I) was observed. Whether rat serum acts in the same capacity as various "activating agents" of the succinoxidase system described by Keilin and Hartree(13) has not been determined. However, Nakamura *et al.*(4) suggested that rat serum may remove or inactivate an 18 carbon fatty acid inhibitor by binding with serum albumin and thus allowing a higher level of oxygen up-

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[†] Obtained from Charles River Breeding Labs, Boston, Mass.

[‡] We are indebted to Dr. Earle Hellerstein who provided the microscopic descriptions.

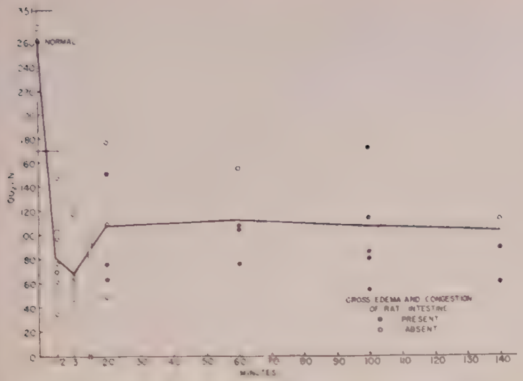

FIG. 1. Effect of endotoxin on succinoxidase activity of rat small intestinal homogenate.

take. Accordingly, measurements of succinoxidase activity in endotoxin treated animals was performed in the presence of 0.3 ml of normal rat serum. Succinoxidase activity decreased 30 seconds after intracardiac injection of endotoxin and remained depressed for 140 minutes (Fig. 1). This decrease was apparent whether the intestine appeared edematous and congested or was normal by gross observation. There was no decrease in heart succinoxidase activity of the same animal throughout endotoxin poisoning (Table II). Average degree of inhibition of heart succinoxidase upon addition of intestinal homogenate in normal rats was 82% (Table II). Endotoxin treated rats exhibited the same degree of inhibition up to 140 minutes after injection (Table II). Addition of varying amounts of endotoxin (from 0.01 ml to 0.5 ml) to Warburg vessels containing normal rat intestinal mucosal homogenates was without effect on succinoxidase activity.

Effect of endotoxin on transmural migration. Since colon and caecum adequately served as their own reservoirs for microorganisms, it was necessary to insure sufficient numbers of microorganisms in the small intestine. Thus, a gastric inoculation technic was used. A 24-hour culture of *Serratia marcescens,* not indigenous to the rat gastrointestinal tract, was grown in nutrient broth (Difco), at room temperature, centrifuged and washed twice with 0.85% saline. The saline washed culture (approx. 3 x 10^8 orgs./ml) was resuspended in 0.1 M potassium phosphate buffer. A series of 25 fasted rats was sacrificed 20, 40, 60 and 100 minutes after endotoxin injection; 15 minutes prior to sacrifice the rats were given 1 ml of the bacterial suspension by stomach tube. A 15 minute interval was used since it was previously determined that *S. marcescens* could be cultured at the ileo-cecal valve 15 minutes after gastric inoculation. Sections of liver, kidney, spleen, lymph nodes and heart were removed aseptically and cultured for 48 and 72 hours in nutrient broth at room temperature The small intestine was also removed and the luminal contents were cultured for *S. marcescens.* These cultures were streaked on nutrient agar plates and incubated for 48 and 72 hours at 25°C.

Neither *S. marcescens* nor intestinal microorganisms could be isolated from heart, liver, kidney, and spleen during acute endotoxin poisoning. Although mesenteric lymph nodes were negative for *S. marcescens,* 40% yielded positive cultures of other microorganisms. Positive cultures for *S. marcescens* were uniformly obtained from the lumen of small intestine. That the failure to grow *S. marcescens* was not due to action of systemic antibacterial defense mechanisms was shown by the following experiment. Eight rats were given 1 ml of endotoxin by intracardiac injection. Forty-five minutes later, they received minimal numbers of these organisms

TABLE II. Per Cent Inhibition of Heart Succinoxidase by Intestinal Homogenate in Endotoxin Treated Rats from 20 to 80 Min. after Inj.

Time after endotoxin inj. (min.)	Qo_2/N			
	Heart	Gut	Heart and gut	% inhibition
20	1031	108	113	89
	898	189	91	99
	960	62	144	85
40	658	76	171	74
	676	51	112	78
	931	233	385	59
60	1366	103	224	83
	729	78	204	72
	900	157	90	90
	1043	104	32	92
80	882	60	94	83
	978	97	345	65
	755	102	79	87
			Mean	= 81 ± 3*
			Control mean†	= 82 ± 4

* Stand. dev.

† 5 animals.

in 0.5 ml of saline, *via* the intracardiac route, (equivalent to approximately 150 orgs./ml of circulating blood). The rats were sacrificed 15 minutes after inoculation with *S. marcescens*. Positive cultures of *S. marcescens* were obtained consistently from heart, liver, kidney, spleen and mesenteric lymph nodes. Thus, at a time when 50% of endotoxin treated rats had gross changes in the small intestine, and all had decreased succinoxidase activity, the animals were unable to clear an intracardiac injection of only 1500 organisms in 15 minutes. Since positive cultures were not obtained under similar conditions when large numbers of *S. marcescens* (3×10^8 orgs./ml) were administered by stomach tube, it is unlikely that *S. marcescens* in numbers greater than 150 orgs./ml of circulating blood passed through the intestinal walls.

Discussion. Although edema and congestion occurred from 20 to 80 minutes following injection of maximal lethal doses of endotoxin, depression of succinoxidase activity was noted 30 seconds after injection of endotoxin. Thus, it appears that the decrease in succinoxidase activity may not have resulted wholly from tissue anoxia, secondary to the vascular reaction, but suggests a more direct effect of endotoxin on the gastrointestinal tract. The fact that inhibitor concentration was not altered while intestinal succinoxidase activity was reduced following endotoxin injection, further supports a direct effect of endotoxin on the enzyme system. These findings are similar to those of Kun and Miller(14), who reported decreases in liver and muscle succinic dehydrogenase activity in rabbits given maximal doses of intravenous endotoxin. Endotoxin, *per se,* had no effect *in vitro* on succinoxidase activity of normal gut homogenate. Furthermore, heart homogenate from endotoxin treated animals did not exhibit a like decrease in succinoxidase activity. Additional study is necessary to clarify this effect. Although the small intestine of intoxicated rats was damaged grossly and altered biochemically, bacterial transmural migration in measurable numbers by viable organisms from gut lumen did not occur. Though not all the defense mechanisms are rendered inoperative by large amounts of endotoxin, it is reasonable to assume there is sufficient impairment of the rat antibacterial defense mechanisms to account for the observed minimal numbers of *S. marcescens* in the tissue. Had viable organisms traversed the intestinal wall into the portal system of the intoxicated rats, these would have been cultured from the visceral organs. The fact that transmural migration did not occur despite morphologic damage and enzymatic impairment may be one indication of the effectiveness of the mucosal barrier.

Summary. 1. Intracardiac injection of maximal lethal doses of endotoxin resulted in grossly apparent edema and congestion and microscopic hemorrhages of small intestine within 20 to 80 minutes. 2. Succinoxidase activity of small intestinal mucosal homogenate decreased within 30 seconds after endotoxin injection and remained depressed until animal was moribund. 3. By assay procedure used, there was no change in amount of succinoxidase inhibitor in the intestinal homogenate. 4. Transmural migration of bacteria through the bowel wall was not apparent despite the effect of endotoxin on gut morphology and enzyme activity.

We wish to acknowledge the generous assistance of Drs. Edward H. Kass and Monto Ho, who also provided the endotoxin, and the valuable suggestions of Dr. Joseph J. Vitale.

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Adsorption Characteristics of Influenza Viruses to Cholesterol and Fatty Acids. (24697)

ALLEN F. WOODHOUR AND KEITH E. JENSEN (Introducd by J. Warren)
Biologics Research Dept., Chas. Pfizer & Co., Terre Haute, Ind.

A new and interesting phenomenon, viral combination with cholesterol(1) and other lipids(2) in chromatographic columns was recently discovered by Youngner and Noll. This reaction might be applicable to preparation of improved influenza vaccines since virus was reported to be adsorbed to cholesterol from infected allantoic fluids, whereas nonviral material was not. Thus, passage of infected chorioallantoic fluid through suitable columns of cholesterol resulted in 15-fold purification of virus. Addition of cholesterol to the biologic might be desirable also since Youngner and Noll presented evidence that inoculation of animals with the cholesterol-influenza complex induced elevated antibody responses by adjuvant action. Our work amplifies these basic studies and in particular describes marked differences in combining capacity with cholesterol which exist for several strains of influenza virus.

Methods. Chromatographic columns were prepared by grinding cholesterol to fine powder with mortar and pestle. Weighed amounts of cholesterol (5 g) were then made into a paste-like consistency with phosphate buffered saline, pH 7.2 (PBS); quantitatively transferred to glass columns and packed with slight positive pressure and repeated washings with PBS. Care was exercised to prevent drying or cracking of columns. Virus suspensions of known concentration were then introduced into the columns and effluent fractions assayed for hemagglutinin (HA) titer with standard pattern tests using 0.5% chicken erythrocytes(3). Cholesterol-virus slurries were prepared using weighed amounts of powdered cholesterol to which virus suspensions of known concentration and quantity were added with grinding in mortar and pestle. The mixture was transferred to Erlenmeyer flasks and incubated at room temperature 30 minutes; spun in refrigerated centrifuge at 1500 rpm 30 minutes and supernate assayed for HA titer. Adsorption efficiency was compared on the basis of either total HA units/g cholesterol or % retained.

Results. Early experiments with crude infected allantoic fluid made it clear that the virus-cholesterol system contained complex variables since the affinity of adsorption to cholesterol differed markedly among strains of influenza virus (Table I). Among type A strains, a gradient was found with Asian isolate, "Formosa," readily combining with cholesterol, whereas other A strains showed decreased affinity. Type B strain, Great Lakes (B/GL), did not combine with cholesterol to any measurable degree. When this type of experiment was repeated with strains partially purified by centrifugation and resuspension in PBS, the virus preparations behaved quite differently In particular, a significant increase was observed in retention value with A/PR 301 and B/GL strains

Additional investigations were carried out with these 2 strains which had demonstrated a marked difference in adsorption rate when partially purified Formosa strain allantoic fluid was separated from virus by high-speed centrifugation and used as suspending medium for sedimented B/GL strain virus. Similarly, sedimented B/GL virus was resuspended in its own allantoic fluid and in PBS. The same rate of retention was found for B/GL virus when suspended in any of above

TABLE I. Cholesterol Retention of Influenza Viruses from Allantoic Fluid or Buffered Saline.

	HA units/g		% virus	
Virus strains	Allantoic fluid	PBS*	Allantoic fluid	PBS
A/Asian/Formosa/57	3200†	3976	100	96
A/PR8/34	7600	10816	59	65
A/PR301/54	1380	4096	43	100
B/GL/54	0	2850	0	68

* Phosphate-buffered saline, pH 7.2.
† Hemagglutinating units with 0.5% chicken erythrocytes.

menstrua (Table II). In contrast, A/PR 301 strain, subjected to similar treatment, was most readily adsorbed when it had been resuspended in PBS. Suspensions prepared with centrifugally sedimented pellets of this virus in either its own original allantoic fluids, or fluid which once contained Formosa virus, were retained by cholesterol columns to the same degree.

When virus suspensions were recycled through fresh cholesterol columns, percentage adsorbed remained constant with PR 8 as shown by Youngner and Noll(1), while with B/GL suspensions, a decrease in adsorption was noted with secondary percolates (Table III). Lack of adsorption with B/GL effluents during second passage suggested that results obtained during the first passage were due to mechanical trapping of aggregates present in original suspensions. To confirm this conclusion, a column of cholesterol was saturated with B/GL strain virus and subjected to usual saline washing procedures. This was continued until wash fractions were free of demonstrable virus by the HA test. Ten ml of saline were then introduced and the column mechanically agitated by stirring.

TABLE II. Absorption of B/GL and A/PR 301 Strains by Cholesterol from Allantoic Fluids or Saline Suspensions.

Virus strain	Suspending medium	% HA units retained
B/GL	As harvested	0
	Resuspended in PBS	42
	Idem Formosa harvest fluid	40
	" B/GL harvest fluid	49
PR 301	As harvested	43
	Resuspended in PBS	100
	Idem Formosa fluid	53
	" PR 301 "	54

This procedure resulted in complete release of virus from the column. Data derived from such experiments with B/GL and other viruses are illustrated in Fig. 1. It was evidently desirable to conduct further experiments by mixing the lipid with virus suspensions in a slurry. These were then clarified by centrifugation and supernatant fluids assayed for virus. Comparison of results with the cholesterol slurry and column methods is provided in Table IV. The most striking differences were seen with allantoic fluid virus suspensions of PR 301 and PR 8 strains. Significant amounts of each virus were retained by cholesterol columns, while no adsorption was noted when viruses were mixed with cholesterol in slurries. In contrast, PR 301 and PR 8 viruses suspended in saline readily combined with cholesterol either in slurry or column. These results suggest that inhibitors in allantoic fluid were most apparent using the slurry method.

TABLE III. Per Cent Retention of Influenza Virus by Cholesterol Columns in Series.

	Virus strain	
Cycle	PR8	B/GL
1	59	39
2	50	6

Youngner and Noll(2) found that other lipids such as aliphatic fatty acids will also absorb viruses, and we have corroborated their observations with additional influenza strains. Table V presents summary of observations with palmitic and stearic acids. In general, higher retention values for all strains were obtained with palmitic acid than with cholesterol, while stearic acid bound a greater amount of virus than did palmitic acid under identical conditions.

Experiments were performed in mice and

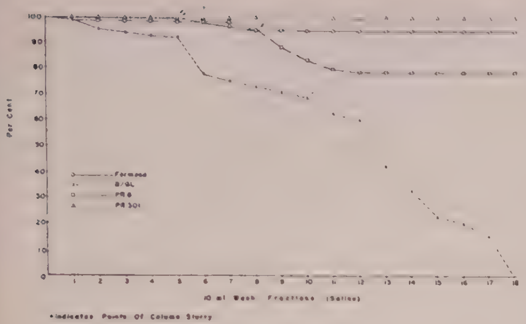

FIG. 1. Per Cent Retention of concentrated influenza viruses on cholesterol columns during successive washings.

[illegible] guinea pigs to establish degree of antibody enhancement in animals which had been injected with influenza virus suspensions to which varying amounts of cholesterol or stearic acid had been added. With such mixtures, not all virus antigens in a polyvalent influenza vaccine would be bound to the lipid. It was of interest to learn whether a cellular response at site of injection was of basic importance or whether virus-cholesterol complexes were necessary to obtain an adjuvanted response. Data from guinea pig experiments where hemagglutinin-inhibiting (HI) antibody was measured with PR8 and B/GL strains are shown in Table VI. These animals were injected with 1 ml of standard 4 strain commercial 1958 polyvalent vaccine diluted 1:5 and, therefore, received only 20 CCA (chick cell agglutinating units) each of PR8 and B/GL components. Average antibody titers obtained were low. However, groups which received 1 ml of vaccine mixed with 5 mg cholesterol or stearic acid developed 2-fold greater antibody titers as measured with PR8 while responses against B/GL generally were very low. Adjuvant effects observed with PR8 at 3 weeks were not evident after 6 weeks. Youngner and Noll(1) reported striking enhancement of antibody responses but a greater proportion of virus in their preparation had been adsorbed to cholesterol.

TABLE IV. Adsorption of Influenza Strains to Cholesterol by Slurry and Column Method.

Method	Virus strain	% virus retention Allantoic fluid	PBS
Slurry	PR 301	0	100
Supernate assay	Formosa	93	100
	PR8	0	75
	B/GL	0	0
Column	PR 301	43	100
Effluent assay	Formosa	100	96
	PR8	59	78
	B/GL	0	0

TABLE V. Absorption of Influenza Strains by Lipids Using the Slurry Method.

Adsorbent	Virus strain	% virus retention Allantoic fluid	PBS
Cholesterol ($C_{27}H_{46}O$)	PR8	0	75
	PR 301	0	100
	Formosa	93	"
	B/GL	0	0
Palmitic acid ($C_{16}H_{32}O_2$)	PR8	61	81
	PR 301	81	100
	Formosa	97	94
	B/GL	0	25
Stearic acid ($C_{18}H_{36}O_2$)	PR8	50	100
	PR 301	93	"
	Formosa	98	"
	B/GL	50	75

TABLE VI. Antibody Responses in Guinea Pigs to Polyvalent Influenza Vaccine Mixed with Cholesterol or Stearic Acid.

		Mean titers* with test strains			
		PR8		GL	
Route of inj.	Adsorbent (5 mg/ml)	3 wk	6 wk	3 wk	6 wk
Subcut.	None	5	8	3	0
	Cholesterol	10	0	0	0
	Stearic	13	10	5	0
Intramusc.	None	6	4	2	0
	Cholesterol	16	4	8	0
	Stearic	13	3	5	0

* Hemagglutination-inhibiting antibody titers in sera from 5 or 6 animals/group.

Discussion. It has long been known that allantoic fluid contains inhibitors for hemagglutination and that certain strains of influenza virus possess enzymes capable of destroying such inhibitors(4). Therefore, differences in adsorption to cholesterol suggested that materials contained in allantoic fluid might differentially affect this adsorption. When virus strains were partially purified by resuspension in PBS, there was a significant increase in adsorption with A/PR 301 and B/GL strains, with the former going from 43 to 100% and the latter from 0 to 68% retention. Further studies indicated 2 factors were operating. One was clumping which resulted from centrifugation, the other was inhibitory activities in allantoic fluid. B/GL virus will not combine with cholesterol. The apparent retention was due to mechanical filtration of clumps from saline suspensions by the cholesterol columns. Composition of type B strain is clearly different since it does not combine with cholesterol as do type A viruses. In contrast, adsorption of A/PR 301 strain to cholesterol is affected by inhibitory materials in allantoic fluids. Adsorption increased when this virus was resuspended in PBS as shown by results from either column or slurry experiments. It is also apparent that Formosa allantoic fluids are not free from inhibitory substances because PR 301 strain resuspended in those fluids did not readily combine with cholesterol. An analogous set of reactions occurs with these 2 strains in HI tests with serum which contains non-specific inhibitory substances(5). Here again, the Formosa strain is completely resistant and readily agglutinates erythrocytes, whereas with the A/PR 301 strain, the hemagglutination pattern will not form in presence of serum inhibitors.

With fatty acids tested the inhibitor content of allantoic fluid observed in cholesterol reactions did not appear to play as significant a role. In contrast to its behavior with cholesterol, type B/GL strain readily combined with stearic acid.

Summary. Marked differences were noted among strains of influenza with respect to adsorption to powdered cholesterol, palmitic acid and stearic acid. It has been shown that some strains suspended in allantoic fluids adsorb to lipids less efficiently than when suspended in buffered saline. Behavior of type B/GL strain was significantly different from type A strains, especially with cholesterol or palmitic acid as adsorbent. Mixtures of minimal amounts of influenza virus with cholesterol or stearic acid elicited an enhanced antibody response in animals when tested at 3 weeks.

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Serum Proteins, Lipoproteins and Glycoproteins. II. Muscular Dystrophy and Related Diseases in Patients.* (24698)

HANS OPPENHEIMER, SHIRILYN SHULMAN, SOPHIE ROBERTS AND ADE T. MILHORAT
Dept. of Medicine, Cornell University Medical College, Russell Sage Inst. of Pathology, and N. Y. Hospital, N. Y. City

Biochemical changes in various forms of muscular dystrophy have been summarized(1, 2). They include changes in serum proteins but in such investigations in clinical muscular dystrophy, classification of patients on the basis of commonly recognized forms of the disease was not always made uniformly or stated clearly. These uncertainties may account for some discrepancies in results. In this report, various forms of muscle disease were strictly differentiated from each other in tabulation of results. The largest group comprised children with pseudohypertrophic muscular dystrophy (PMD). Other muscle diseases investigated were myositis, myotonia dystrophica and amyotrophic lateral sclerosis. Some patients with scoliosis and with Perthes disease also were studied, since sometimes muscular wasting was present which was of secondary nature. The purpose of this investigation was to find significant changes of serum proteins, lipoproteins and glycoproteins

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* Supported by grant from Muscular Dystrophy Assn.

in patients with muscle diseases, and to compare the results with our findings in similar, previous studies on muscular dystrophy of vit. E-deficiency in rabbits(3).

Methods. Blood samples were taken by venipuncture from fasting subjects. Serum was separated by centrifugation soon after clotting and usually worked up within a few days, during which period it was kept at about +3°C (in case of very short storage) and otherwise at –15°C. Serum proteins, lipoproteins and glycoproteins in barbiturate buffer of pH 8.6 and of ionic strength 0.1 were fractionated by both moving boundary and paper electrophoretic methods. The procedures for these technics were generally the same as described previously(3). Concentrations of total protein, total and free cholesterol, phospholipids and protein-bound hexoses, hexosamines and sialic acid also were determined by previous methods(3). Furthermore, by differential elution of paper electrophoretic lipoprotein fractions with acetone and petroleum ether, total phospholipids and sphingomyelins were obtained(4). Protein-bound fucose was determined by the cysteine method(5). The data were evaluated statistically by Student's "t" test(6).

Results. The Tables show mean values with standard deviations. Since all patients with PMD, scoliosis and Perthes disease were children, they were compared with healthy children as controls. However, patients suffering from myositis, myotonia dystrophica and amyotrophic lateral sclerois were adults and, therefore, were compared with healthy adults as controls. Those mean values which differed from corresponding ones of their appropriate controls on the statistically significant level of 1% and on borderline level of 2% are underlined in the Tables. Numbers in parentheses are numbers of subjects whenever they differed from those given in second column of Tables.

Table I shows relative concentrations of serum protein fractions in moving boundary electrophoretic patterns. Albumin decreased in myositis and scoliosis and α_2-globulin increased in all muscle diseases. (The increase in case of amyotrophic lateral sclerosis, however, could not be shown to be really significant, presumably because of the small number of cases.) Other globulins did not vary significantly, with the exception of increase of γ-globulin in scoliosis. Total protein concentration did not change significantly and electrophoretic mobilities for corresponding fractions were alike in the various groups.

Paper electrophoresis showed relative concentrations of protein fractions alike in patients and controls except for increase of β-globulin in PMD on a 2% level of significance only. In scoliosis, however, albumin was lowered and γ-globulin increased, as in moving boundary electrophoresis; β-globulin increased on a 2% level of significance.

Relative concentrations of total protein-bound lipids in paper electrophoretic fractions in the various diseases did not differ much from those of corresponding controls (Table II), but there was a decrease of α-lipoprotein on the 2% level of significance in PMD and

TABLE I. Moving Boundary Electrophoresis of Serum Proteins in Muscle Diseases.

		Relative concentrations				
	No. of subjects	Albumin	α_1-globulin	α_2-globulin	β-globulin	γ-globulin
		%				
Pseudohypertrophic M.D.	17	52.7 ± 2.4	7.0 ± .8	11.7 ± 1.1	14.7 ± 1.8	13.9 ± 2.2
Myositis	9	46.6 ± 2.6	7.9 ± 1.1	12.1 ± 2.1	15.9 ± 1.7	17.5 ± 4.9
Myotonia dystrophica	5	46.3 ± 10.3	7.1 ± 1.0	12.5 ± 1.4	17.4 ± 3.4	16.7 ± 10.5
Amyotrophic lateral sclerosis	3	54.9 ± 4.7	7.0 ± 1.2	12.0 ± 2.3	15.9 ± 1.2	10.2 ± 7.7
Scoliosis	6	49.9 ± 1.8	7.2 ± 1.2	10.8 ± .9	13.9 ± 1.0	18.2 ± 2.4
Perthes disease	3	52.0 ± 3.5	6.9 ± 1.2	11.3 ± .6	14.1 ± 2.6	15.7 ± 3.2
Normal children	8	55.7 ± 3.0	6.65 ± .6	10.3 ± .9	14.7 ± 1.3	12.65 ± 3.4
" adults	10	53.9 ± 2.8	7.4 ± .8	8.9 ± 1.0	13.9 ± 1.9	15.9 ± 1.9

Underlined values, $P < .01$.

TABLE II. Serum Protein-Bound Lipids in Muscle Diseases.

		Paper electrophoresis of lipoproteins			Cholesterol ratio
		Relative conc.			
	No. of subjects	α-l.p.	β-l.p.	"O" l.p.	Free/Total
		%			
Pseudohypertrophic M.D.	16	35.7 ± 7.2	44.1 ± 6.8	20.2 ± 7.5	24.6 ± 3.7 (17)
Myositis	8	28.2 ± 6.8	46.8 ±10.4	25.0 ±11.7	22.2 ± 3.8 (4)
Myotonia dystrophica	3	28.2 ±10.2	56.0 ± 2.5	15.8 ± 8.7	23.8 ± 4.4
Amyotrophic lateral sclerosis	3	35.7 ± 4.7	39.7 ± 1.7	24.5 ± 3.9	
Scoliosis	6	33.1 ± 7.3	44.3 ± 8.4	22.6 ± 8.9	27.8 ± 3.1
Perthes disease	3	30.6 ± 6.1	44.7 ± 8.6	24.7 ±13.6	27.4 ± 2.2
Normal children	9	42.3 ± 5.5	43.8 ± 6.1	13.9 ± 5.5	20.7 ± 3.1 (7)
" adults	10	33.2 ± 7.6	43.2 ± 4.2	23.6 ± 7.5	25.8 ± 2.3 (8)

Underlined values, P <.01. Broken underlined values, P <.02.

definite increase of β-lipoprotein in myotonia dystrophica. Distribution of both total phospholipids and sphingomyelins in these lipoprotein fractions was similar in all groups. The absolute concentrations of total phospholipids and cholesterol did not vary significantly nor did the ratio of the two. The Table shows, however, that the ratio of free to total cholesterol increased in PMD (on the 2% level of significance), scoliosis and Perthes disease.

Table III lists absolute concentrations of protein-bound carbohydrates. Total hexoses were decreased in PMD, scoliosis and Perthes disease and increased, on a 2% level of significance, in myositis. Amounts of hexoses in the seromucoid fraction were similar in all groups and their ratios to total hexoses were fairly constant. Hexosamines varied only in Perthes disease in which they were decreased. Sialic acid was the only protein-bound carbohydrate which increased significantly in PMD. Its apparent elevation in other muscle diseases was not of statistical significance because of large deviations and small numbers of subjects. Fucose, a minor constituent, followed the same trend. The percentile distribution of total protein-bound carbohydrates among paper electrophoretic fractions was alike in all groups.

Discussion. Various changes of relative concentrations of serum protein fractions in electrophoretic patterns in muscle diseases have been reported(7-12). Dryer, *et al.*,(8) found increase of α_2-globulin in moving boundary electrophoresis in PMD, but, unlike our results, decrease of γ-globulin. They described twinning of the α_2-globulin peak as a nearly regular and characteristic feature of the disease, but we observed this only occasionally. The peak was usually asymmetric and broad, indicating electrophoretic heterogeneity. The increase in relative concentration of α_2-globulin resulted from an increase of conjugated non-protein constituents rather than of proteins themselves, since the latter did not change in paper electrophoretic patterns. It probably was related to the increase of sialic acid which has high affinity for α_2-globulin(13,

TABLE III. Serum Protein-Bound Carbohydrates in Muscle Diseases.

	Hexoses	Hexosamines	Sialic acid	Fucose
	mg %			
Pseudohypertrophic M.D.	97.6 ±15.9(24)	99.5 ± 9.4(14)	77.5 ±15.8(17)	10.1 ± 4.4(16)
Myositis	126.5 ±20.2 (5)	110.5 ±17.1 (5)	84.3 ±16.7 (5)	12.3 ± 4.0 (3)
Myotonia dystrophica	105.7 ±22.9 (3)	127.0 ±12.6 (3)	93.1 ±17.6 (3)	12.7 ± 6.2 (3)
Scoliosis	82.5 ± 8.9 (6)	107.9 ± 8.4 (5)	65.5 ± 8.5 (6)	10.8 ± .6 (4)
Perthes disease	75.7 ± 4.6 (3)	92.3 ± 2.3 (3)	69.1 ± 4.0 (3)	8.3 ± 2.6 (3)
Normal children	121.6 ±11.1 (9)	106.6 ± 5.7 (9)	59.3 ± 8.0 (9)	9.5 ± 1.6 (8)
" adults	98.1 ± 9.4 (8)	104.1 ±10.3 (7)	68.1 ±10.7 (8)	8.4 ± 2.0 (8)

Underlined values, P <.01. Broken underlined values, P <.02.

14). It was not specific for muscle diseases, and has been found in other wasting diseases (5).

Diseases with tissue destruction are usually accompanied by high levels of sialic acid, and of the 2 other main protein-bound carbohydrates, hexoses and hexosamines(5). In muscle diseases, however, these 2 constituents did not increase significantly. Since the value for total hexoses in normal adults was lower than usual(5,15), the moderate increase of this substance in myositis may be only apparent. In PMD total hexoses actually decreased, which may be related to their reported low value in cerebrospinal fluid(16).

In contrast to our results Dryer *et al.*,(8) found the level of phospholipids in PMD decreased to less than half normal value. Total cholesterol has been reported to decrease(17), but we found such a decrease only on a 5% level of significance. Similar studies of muscular dystrophy of vit. E-deficiency(3) had revealed decrease of albumin and increase of β-globulin in moving boundary electrophoretic patterns. The rise of β-globulin was related primarily to strikingly elevated levels of cholesterol and phospholipids. Such severe disturbance of lipid metabolism was reflected also by marked shift in distribution of lipoproteins in paper electrophoretic patterns. Another difference between this nutritional myopathy and clinical forms of the disease was the increase, in the former, of all main protein-bound carbohydrates.

Summary. 1. Serum proteins, lipoproteins and glycoproteins were studied primarily in pseudohypertrophic muscular dystrophy (PMD), myositis, myotonia dystrophica and amyotrophic lateral sclerosis, and in scoliosis and Perthes disease with occasional secondary muscular changes. 2. Moving boundary electrophoretic patterns revealed significant, although not specific, increase of α_2-globulin in nearly all muscle diseases. This increase seemed to be related to a high level of sialic acid, particularly in PMD. 3. Distribution of lipoproteins in paper electrophoretic patterns and concentrations of cholesterol and phospholipids did not show the pronounced changes previously found in muscular dystrophy of vit. E-deficiency in rabbits. 4. In contrast to this experimental myopathy and many other wasting diseases, human muscle diseases were not accompanied by significant increase of protein-bound carbohydrates other than that of sialic acid in PMD.

The authors gratefully acknowledge helpful cooperation of the following, in obtaining blood sera: patients with muscle disease in metabolism ward and day school at Payne Whitney Clinic and Out-patient Dept. N. Y. Hosp.; children with scoliosis at Hosp. for Special Surgery; children with Perthes disease at St. Agnes Hosp., White Plains, N. Y.; children at Glenwood Community Center, Brooklyn, N. Y. and its Director, M. Eisenstein; colleagues at Payne Whitney Clinic; Dr. Virginia Weeks and Dr. Leon I. Charash, N. Y. Hosp.; Dr. Melville H. Manson and Miss Frances Morrow, Muscular Dystrophy Assn.

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Serum and Plasma Proteins, Lipoproteins and Glycoproteins. III. Hereditary Muscular Dystrophy in Mice.* (24699)

Hans Oppenheimer, Alma de Luca and Ade T. Milhorat
Dept. of Medicine, Cornell University Medical College, Russell Sage Inst. of Pathology, and N. Y. Hospital, N. Y. City

Spontaneous occurrence of genetic mutation resulting in hereditary muscular dystrophy in inbred strain of mice was observed recently(1). This report deals with proteins, lipoproteins and glycoproteins in blood serum and plasma of such mice. The results are compared with those found in similar studies on human muscular dystrophy(2) and on myopathy of Vit. E-deficiency in rabbits(3).

Methods. Dystrophic mice and control litter mates of strain 129 were supplied by Roscoe B. Jackson Memorial Lab., Bar Harbor, Me. They were maintained on Purina Laboratory Chow, and sacrificed at ages 6 to 9 weeks. For determination of protein-bound lipids and carbohydrates, the animals were fasted at least 6 hours, and usually overnight, before blood samples were taken. Blood was obtained by decapitation under ether anesthesia or, occasionally, by cardiac puncture. Serum was used for paper electrophoretic runs, and oxalated plasma for determinations of protein nitrogen and of protein-bound lipids and carbohydrates. Procedures for paper electrophoretic fractionation of serum proteins, lipoproteins and glycoproteins were generally the same as described previously(3). Concentrations of protein nitrogen, total and free cholesterol, phospholipids and protein-bound hexoses, hexosamines and sialic acid also were determined as previously reported(3). Some preliminary determinations of amounts of esterified fatty acids were performed(4). In comparison of results on dystrophic mice with those on control litter mates, differences between corresponding mean values were considered to be statistically significant if p-values were .01 or less. In such cases mean values are underlined in Tables showing control values. For most determinations number of mice used was 10 to 15.

Results. Relative concentrations of serum protein fractions of dystrophic mice did not differ significantly from those for normal litter mates (Table I). The lipoprotein pattern consisted of intense band at location of albumin, (occasionally extending somewhat ahead of the latter), and of a band spread widely among the globulins. The former band showed highly significant decrease to 42.7 ± 8.6% in dystrophic mice ($P < .001$). The other band increased correspondingly, but its diffuse nature did not permit definite differentiation among the various globulins. Total areas of lipid stains were about the same in the 2 groups. The pattern of serum glycoproteins consisted of 3 bands at locations of α_1-, α_2- and β-globulins. The band associated with α_2-globulin was lowered to 40.2 ± 4.8% in dystrophic mice ($P < .01$).

Concentrations, in plasma, of protein nitrogen and of protein-bound lipids and carbohydrates were somewhat lower, but not significantly so in dystrophic mice (Table II). Cholesterol values are similar to those of other workers(5). Ratio of cholesterol to phospholipids was alike in the 2 groups (0.65-0.67).

Discussion. The group of normal litter mates used as controls consisted of both heterozygous and homozygous individuals, depending on type of mating. To exclude the possi-

TABLE I. Paper Electrophoresis of Serum Proteins, Lipoproteins and Glycoproteins of Normal Mice (Strain 129).

	Proteins	Protein-bound Lipids	Protein-bound Carbohydrates
	%	%	%
Albumin	51.6 ± 3.7	$\underline{\underline{59.3 \pm 9.3}}$	
α_1-globulin	6.4 ± 3.2		21.1 ± 8.2
α_2- "	14.3 ± 5.1		$\underline{46.0 \pm 4.5}$
β- "	17.3 ± 3.2		32.9 ± 8.5
γ- "	10.4 ± 2.3		

Single underline, $P < .01$.
Double underline, $P < .001$.

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* Supported by grant from Muscular Dystrophy Assn.

TABLE II. Plasma Proteins and Protein-Bound Lipids and Carbohydrates of Normal Mice (Strain 129).

Protein nitrogen, g %	.838	± .096
Total cholesterol, mg %	132	± 34
Free chol./total chol., %	36.0	± 13.4
Phospholipids, mg %	204	± 50
Esterified fatty acids, mg %	398	±253
Hexoses, "	84.8	± 17.5
Hexosamines, "	86.4	± 20.0
Sialic acid, "	128.4	± 26.7

bility that the heterozygous litter mates were affected by mutation, the same series of determinations was performed with a group of normal homozygous mice. Estimates for the 2 control groups did not differ significantly except for a doubtful decrease in ratio of free to total cholesterol in the homozygous group.

Migration of the main band of stained lipids occasionally ahead of the protein stain for albumin may have been due to prevention by the lipid of staining of the protein.

The significant changes found in this study of dystrophic mice were shifts in patterns of lipoproteins and, to a lesser extent, of glycoproteins. In pseudohypertrophic muscular dystrophy in man changes observed(2) were: increase of α_2-globulin in moving boundary electrophoretic pattern, increase of β-globulin and decrease of α-lipoprotein in paper electrophoretic pattern, increase in ratio of free to total cholesterol and increase of sialic acid. (The first and last changes were at 1% level of significance, the others did not exceed the 2% level.) Myopathy of Vit. E-deficiency in rabbits was characterized by decrease of albumin, increase of β-globulin and lowering of α-lipoprotein together with marked rise of β-lipoprotein(3). This alteration in lipoproteins was assoiated with large increases of both free and esterified cholesterol and of phospholipids. Also the levels of protein-bound hexoses, hexosamines and sialic acid were raised in sera of Vit. E-deficient rabbits, but did not increase in the 2 hereditary forms of dystrophy, with the exception of sialic acid in the human disease. How closely the morbid process in mice resembles the hereditary muscular disease in man or the deficiency state of rabbits is not clear. Lack of large variation in levels of most constituents studied is similar in the 2 hereditary forms of the disease. Distribution pattern of lipoproteins, however, was altered both in hereditary dystrophy of mice and in nutritional dystrophy of rabbits. These 3 forms of muscular dystrophy involve 3 different species so that only cautious comparisons should be made.

Summary. 1. Paper electrophoretic patterns of sera from mice with hereditary form of muscular dystrophy and from normal litter mates as controls showed no change in relative concentrations of the protein fractions themselves, but did indicate a shift in distribution particularly of lipoproteins and, to a smaller extent, of glycoproteins. 2. Concentrations, in plasma, of protein nitrogen, protein-bound lipids (free and esterified cholesterol, phospholipids, esterified fatty acids) and carbohydrates (hexoses, hexosamines and sialic acid) did not vary significantly from those of controls. 3. Results have been compared with those from previous, similar studies on clinical forms of muscular dystrophy and on myopathy of Vit. E-deficiency in rabbits.

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Comparison of 3 Chelating Agents in Treatment of Experimental Manganese Poisoning[*] (24700)

JOAN F. FRIED, ARTHUR LINDENBAUM AND JACK SCHUBERT
(Introduced by A. M. Brues)
Division of Biological and Medical Research, Argonne National Lab., Lemont, Ill.

Ethylenediaminetetraacetic acid, (EDTA), has been tested in treatment of acute manganism of laboratory animals(1,2) and has been recommended for clinical use in the industrial disease(3). It has been suggested that the relative effectiveness of a chelating agent, A, for manganese binding under physiological conditions can be related to the value of the ratio $\frac{K_{MnA}}{K_{CaA}}$(4). This ratio, as shown below, is more favorable in the case of diethylenetriaminepentaacetic acid (DTPA) than for EDTA, indicating that DTPA could be superior to EDTA for treatment of Mn poisoning. In addition, a third compound, ethylene bis (α - imino - o - hydroxyphenylacetic acid)[†] (EBAA) was of interest. While K_{MnA} and K_{CaA} for this compound were not determined, it was known the value of K_{CaA} was considerably smaller than that of EDTA and it was hoped that the net value of $\frac{K_{MnA}}{K_{CaA}}$ would be greater than that for EDTA. Ability of DTPA and EDTA to protect against manganese poisoning was tested in two ways: (a) survival studies on rats given an LD_{90} of manganese gluconate and treated at varying times afterward with either CaEDTA, CaDTPA or saline; and (b) survival studies on rats given the manganese chelates of EDTA and DTPA. The effect of EBAA administration was studied by means of a tissue distribution experiment in which animals were given a tracer dose of Mn^{54} and treated with EBAA, EDTA, or saline, and by a survival experiment in which animals were given an LD_{90} of stable manganese and treated 75 minutes later with EBAA.

Methods. Female Sprague-Dawley rats between 70 and 135 days of age, weighing 190-280 g, were used; animals of equal age were selected for each experimental group. In the survival studies manganese was administered intraperitoneally as a 3.5% solution of manganous gluconate, pH 6.2, at 27 mg/kg as Mn, a dose which had previously been determined to be the 30-day LD_{90} value. The calcium and manganous chelates of EDTA and DTPA were prepared by neutralizing the acid or sodium salt with NaOH, adding calcium or manganous gluconate in 1:1 molar ratio of metal to chelating agent, and adjusting the concentration to 0.16 *M* and the pH to 7.2. CaEDTA and CaDTPA were injected into a tail vein or intraperitoneally in equimolar ratios at levels of from 105 to 2000 mg/kg; doses of these compounds, wherever given, are expressed as the acid form of the chelating agent. MnEDTA and MnDTPA were injected intraperitoneally in doses providing 130 to 260 mg/kg as Mn. EBAA was injected intravenously as the tetrasodium salt at pH 8.5. All survival data given are on a 30-day basis, but surviving animals were kept under observation for 90 days. In the tissue distribution studies, $Mn^{54}Cl_2$ was given intravenously as carrier-free solution to 4 groups of 3 rats each, at a level of 1 μc per rat. These groups were then given intravenous injections of the chelating agents at 1 hr after Mn^{54} injection, and on the following 2 days as follows: (a) EBAA at 80, 40 and 40 mg/kg, (b) CaEDTA in equimolar ratio to this (65, 32.5 and 32.5 mg/kg), (c) CaEDTA at 200 mg/kg per injection

TABLE I.

A	Log K_{MnA}	Log K_{CaA}	Log $\frac{K_{MnA}}{K_{CaA}}$
DTPA(5,6)[*]	15.1	10.6	4.5, 5.0
EDTA(6,7)	13.5, 13.6, 14.0	10.6	2.9, 3.0, 3.4

[*] Values for these constants are in agreement with those obtained from H. Kroll (personal communication, June, 1956).

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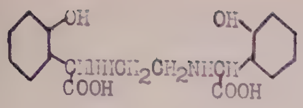
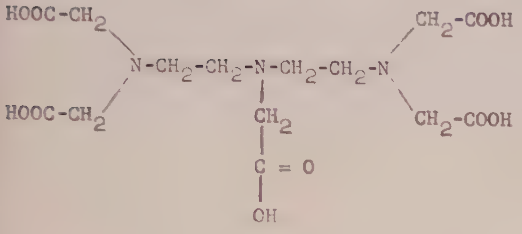
[*] This work performed under the auspices of U. S. Atomic Energy Commission.

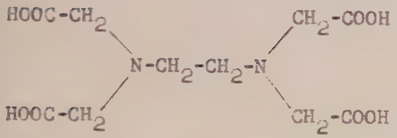
[†] Supplied through courtesy Geigy Chemical Corp.



EBAA



DTPA



EDTA

and (d) saline in equivalent volume. The rats were sacrificed on the fourth day and the following tissues removed: femurs, brain, pancreas, liver, kidneys and blood. These, and the daily collections of urine and feces, were disintegrated with concentrated nitric acid. The tissues and urines were counted in a NaI (T1) well counter in conjunction with a single channel, pulse height analyzer at the optimum setting for the 0.84-MEV γ-ray. Feces and carcasses were counted in a pressurized ionization chamber designed to permit 100% geometry and sensitive to 0.06 μc Mn^{54}.

Results. Toxicity studies. Intraperitoneal administration of CaDTPA at a level of 2000 mg/kg resulted in 90% survival, while, at a corresponding equimolar level of CaEDTA of 1490 mg/kg, survival was 100% (20 rats per group). However, 70% of the CaDTPA-injected animals at this dose level developed paralysis of the hind limbs on approximately the 6th day after injection; this was of several weeks duration with partial recovery in 2 months. At a slightly lower level (1830 mg/kg) of CaDTPA no deaths or paralyses occurred. Intravenous administration of CaDTPA in amounts up to 250 mg/kg produced no toxic effects. The toxicity data obtained on EBAA were not extensive; however, it was established that 70 mg/kg could safely be given daily for 3 days by the intravenous route.

Survival studies. Equimolar amounts of CaDTPA (158 mg/kg and CaEDTA (118 mg/kg) gave complete protection when injected intravenously as long as 3½ hours after administration of an LD_{90} of manganese gluconate. However, at 5 hours after manganese administration, they were only partially effective (50% survival). Average time to death of non-survivors was longer for the DTPA-treated as compared with the EDTA-treated. In another experiment, the minimum effective dose for complete protection against an LD_{90} of manganese gluconate, when given 1 hour after the manganese, was found to be 115 mg/kg for each of the 2 compounds.

Survival data from rats given the manganese chelates of DTPA and EDTA intraperitoneally are shown in Table II. When manganese is administered as the DTPA chelate, a higher dose may be given without toxic effects than when it is administered as the EDTA chelate.

There was no significant difference in survival of a group of 10 rats given an LD_{90} of manganese gluconate and treated 75 minutes later with 60 mg/kg EBAA intravenously as compared with that of the untreated control group.

Tissue distribution studies. No significant differences were present in distribution of carrier-free Mn among the tissues of the EBAA treated animals as compared with the saline controls (Table III). The route and extent

TABLE II. Survival of Rats Given Manganese Chelates of DTPA and EDTA.

Dose of Mn, mg/kg	Survival, %	
	MnDTPA	MnEDTA
130	100 (10)*	100 (10)
162.5	100 (6)	83 (6)
195	100 (17)	70 (17)
225	94 (16)	60 (10)

* No. of animals inj. are in parentheses.

TABLE III. Effect of EBAA and EDTA on Manganese Distribution and Excretion in Rats.

	Mn^{54} content in % inj. dose*						
Treatment	Both femurs	Carcass	Pancreas	Both kidneys	Liver	Urine	Feces
EBAA 80, 40, 40 mg/kg	.25 ±.02	21.8 ±1.3	2.30 ±.27	2.28 ±.35	15.5 ± .8	†	70.1 ±4.83
EDTA 65, 32.5, 32.5 mg/kg	.22 ±.04	21.1 ± .3	2.89 ±.13	1.71 ±.08	17.3 ±1.0	5.87±.96	59.6 ± .8
EDTA 200, 200, 200 mg/kg	.20 ±.03	21.7 ± .5	2.30 ±.15	1.40 ±.17	15.4 ± .9	15.2 ±.2	46.0 ± .1
Saline	.21 ±.02	24.1 ±3.2	2.78 ±.15	2.10 ±.20	14.6 ± .9	†	61.7 ±6.3

* Each value represents mean from 3 rats ± stand. error of mean. Time of sacrifice was 4 days after inj. with Mn.

† Too low to count (less than 2 times background).

of Mn excretion were also unaltered by the EBAA treatment. An equimolar dose of CaEDTA produced a small but significant increase in excretion of Mn in the urine with a corresponding decrease in the feces. The larger dose of CaEDTA promoted a greater shift in the excretion pattern from feces to urine, and lowered the Mn content of the kidneys by about 20%. Neither compound had any effect on quantity of Mn retained in the liver or on total retention of injected dose.

Discussion. The data presented in Table III show little difference in retention of carrier-free Mn between the rats given EDTA and the controls. However, in the case of animals given Mn in large, toxic doses, it has been shown(2) that the liver burden is markedly reduced in EDTA-treated groups. This, together with the reduction in the kidney content, is the basis of the protection afforded by EDTA and DTPA, since animals dying of acute experimental manganese poisoning show marked pathology of liver and kidney.

The primary excretory route of intravenously or intraperitoneally injected manganese is fecal, mainly *via* the bile(8). It has been shown that manganese is excreted in the feces at 2 rates. The faster presumably represents the unbound manganese and the slower represents that portion which becomes bound in tissues, predominantly in the cells of liver, kidney and pancreas(8) and is then slowly released. There is experimental evidence to suggest that EDTA competes successfully with the body tissues for some of the manganese which would otherwise become bound thereby increasing the amount released in the fast component(8). This is accompanied by a shift in excretion of manganese from the feces to an earlier appearance in the urine(8). Presumably DTPA has the same mode of action.

Both CaEDTA and CaDTPA are beneficial in treatment of experimental, acute manganese poisoning. However, there are some indications that CaDTPA is more effective: (a) more manganese may safely be given as the DTPA chelate than as the EDTA chelate, (b) although no significant differences in survival numbers were present when CaDTPA and CaEDTA were given several hours after manganese injection, the time to death of the nonsurvivors was significantly longer in the case of the CaDTPA treated group, and (c) the dose of CaDTPA needed for protection is smaller, on a molar basis. The titration data obtained in this laboratory(9) indicate that the enhanced effectiveness of CaDTPA is due to greater stability of MnDTPA.

DTPA is far more effective in decreasing the body burden of trivalent and tetravalent elements than is EDTA(10,11,12,13). This is to be expected, since the stability constant ratios $\frac{K_{MA}}{K_{CaA}}$ of DTPA for these metals exceed those of EDTA by factors of 10^2-10^4, whereas in the case of divalent Mn the increase is only of the order of 10.

The failure of EBAA to protect against manganese poisoning is undoubtedly a reflection of too low a value of K_{MnA}/K_{CaA}.

Summary. Three chelating agents of the polyamino polycarboxylic acid type, ethylenediaminetetraacetic acid, diethylenetriaminepentaacetic acid, and ethylene bis(α-imino-o-hydroxyphenylacetic acid) have been compared in treatment of acute experimental manganese poisoning. EBAA had no therapeutic value, whereas both EDTA and DTPA provided significant protection to rats given lethal amounts of manganese gluconate. It is shown that DTPA is somewhat more effective than EDTA.

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Coronary Arteriosclerosis and Thrombosis in the Rat.* (24701)

Bernard C. Wexler and Benjamin F. Miller
(With technical assistance of Lionel Staples and Janice Doron)
May Inst. for Medical Research, Cincinnati Jewish Hospital and Depts. of Pathology and Medicine, Univ. of Cincinnati College of Medicine

Severe arteriosclerosis has been produced in female rats (Sprague-Dawley) bred repeatedly, unilaterally nephrectomized, and then given ACTH for 4 to 7 weeks (Wexler and Miller, 1). Lesions involve systemic, cerebral and peripheral arteries. Large, flame-shaped hemorrhages and aneurysms are seen in the aortic arch. Particularly conspicuous upon gross examination are the distended and tortuous coronary vessels. Myocardial infarcts are readily visible. Histologically, a spectrum of early to advanced lesions has been observed in coronary arteries. These findings appear to have a special importance because (a) the rat is ordinarily resistant to development of arteriosclerosis(2-5), (b) these rats were not given fat in their diet and had normal serum cholesterol levels, and (c) the pathology in coronary arteries shows some striking resemblances to that observed in human disease.

Materials and methods. Female rats of the Sprague-Dawley strain were (1) bred repeatedly, (2) subjected to unilateral nephrectomy, and (3) given ⅓ unit of ACTH (gel)/100 g body weight, subcutaneously, 3 times/week, for 4 to 7 weeks. The particular strain of rat can be as important as the other 3 factors in production of satisfactory arteriosclerotic lesions. After nephrectomy, animals were allowed 10 days to recover before administration of ACTH. Animals were housed in air-conditioned quarters and given *ad lib.* a regular commercial rat chow and water. No extra fat or salt was added to the diet. At autopsy, the rats were decapitated, exsanguinated, and tissues intended for microscopic examination quickly removed and fixed in 10% buffered neutral formalin (Lillie). Hematoxylin and

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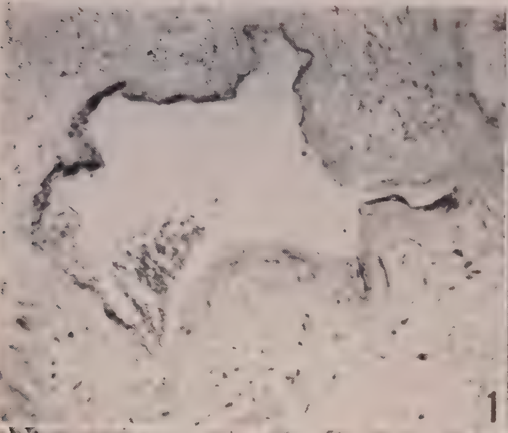
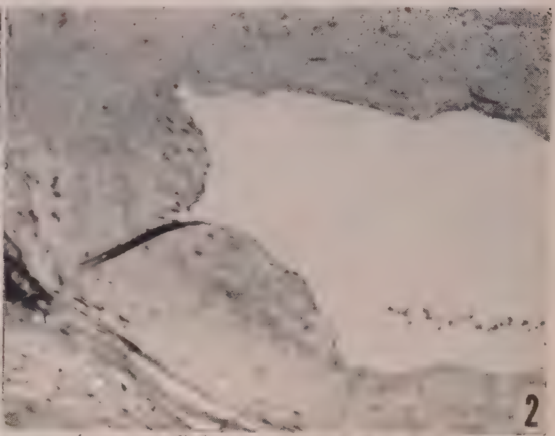
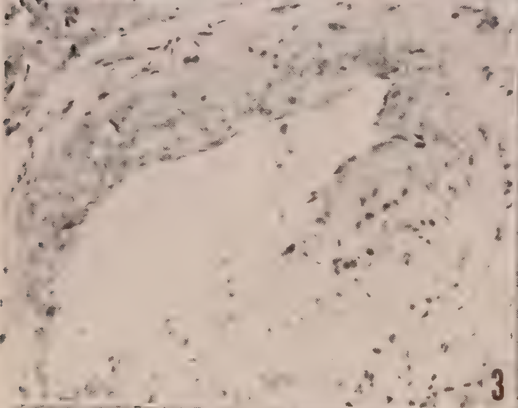
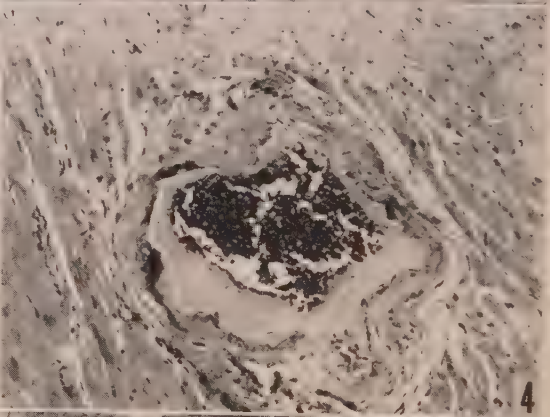
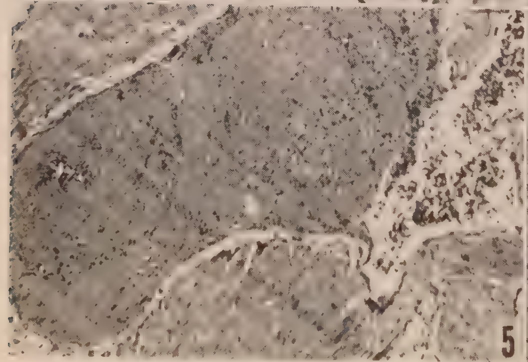










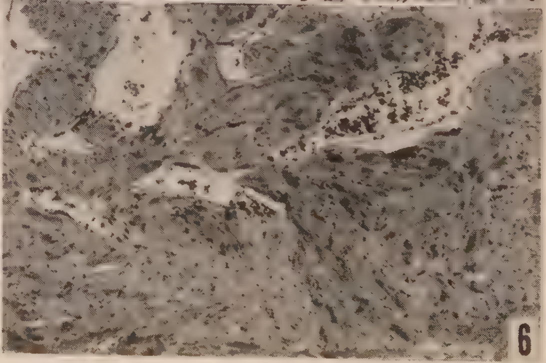


FIG. 1. Main coronary artery and branch showing intimal basophilia and thickening. Female, Sprague-Dawley rat, bred repeatedly, unilaterally nephrectomized, and given ACTH for 7 wk. H & E, ×100.

FIG. 2. Coronary artery of rat treated in same manner showing intimal hyperplasia. H & E, ×500.

FIG. 3. Coronary artery showing more advanced intimal hyperplasia and medial elastosis. H & E, ×180.

FIG. 4. Coronary artery showing early thrombus formation, and intimal and medial basophilia and elastosis. H & E, ×210.

FIG. 5. Mural thrombus formed in atrial wall. Note beginning organization. Myocardial fibers are enlarged. H & E, ×100.

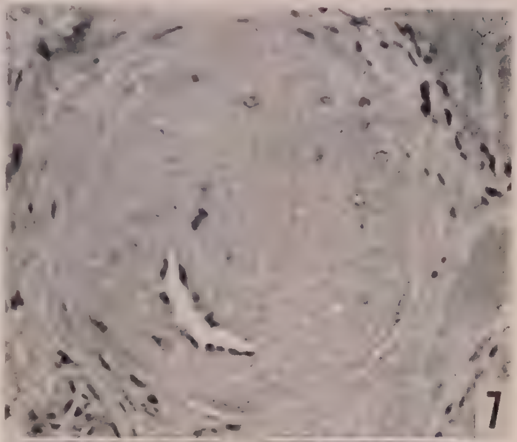
FIG. 6. Myocardial infarct. Light staining areas in lower center and the extreme right represent scarred tissue. Infarcts are most frequently found on endocardial surface of the left ventricle. H & E, ×100.

eosin, Masson and Mallory's connective tissue stain, P.A.S., and Gomori's aldehyde fuchsin stain for elastic fibers were used routinely. Sudan Orange and Sudan Black B were employed for lipids, on frozen sections.

Results. Of 100 rats examined, 75 showed some degree of atherosclerosis of coronary arteries. In this same group, 38 animals displayed significant intimal thickening and derangement of ground substance and connective tissue elements in both intima and media. Similarly, 30 animals manifested myocardial infarction. Microscopic examination showed generalized distribution of arteriosclerotic lesions in the coronary arteries. The larger epicardial branches were most severely involved. Fig. 1 is a cross section of a main coronary vessel and one of its branches, each showing equal involvement. Some lesions consisted of intimal hyperplasia only (Fig. 2) while others displayed intimal basophilia either alone or accompanied by medial elastosis (Fig. 3). We suspect that this basophilic material may be either calcium or mucopolysaccharide material—or a mixture of the two. Thrombi have been found more frequently in medium-sized coronary arteries and less frequently in larger arteries (Fig. 4). The clots exhibited typical appearance of ante-mortem thromboses. Mural thrombi in various stages of organization (Fig. 5) were frequently observed adherent to the atrial wall.

In other areas, coronary arteries show a distinctly different pattern of change. For example, some arteries undergo intimal and medial swelling which caused virtually complete obliteration of the lumen (Fig. 7). This usually occurs in medium-sized arteries. Occasionally, the obliterative type of ingrowth contains giant foam cells (Fig. 8).

Discussion. Virtually all rats so treated developed systemic arteriosclerosis. Approximately 75% of these rats developed coronary lesions. The high incidence of coronary involvement should make this a useful tool in evaluation of preventive and therapeutic procedures. There are many resemblances between the lesions observed in these rats and in humans. Particularly gratifying has been the high rate of true ante-mortem thrombosis with attendant myocardial infarctions. The mural thrombi also closely resemble those in human disease.



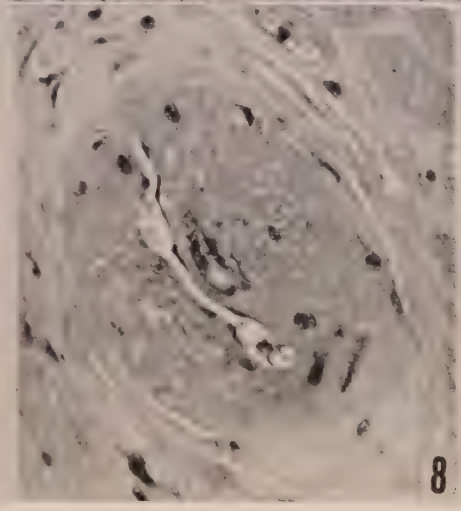


FIG. 7. Coronary artery showing endothelial and medial swelling leading to obliteration of lumen. H & E, ×180.

FIG. 8. Coronary artery. Lumen is closed. Note giant foam cells. H & E, ×180.

The absence of added fat in the diet in our experiments should be noted. Also, there was no elevation in blood cholesterol. However, there may have been changes in other lipids and lipoproteins. Some of these factors are now under study. Only an occasional lesion, and usually an advanced one, showed demonstrable fat (Fig. 8). This condition is the exception rather than the rule.

Sex differences are especially prominent in this form of generalized arteriosclerosis which appears much more readily in the female. For this reason we have restricted this study to female rats. However, recent observations suggest that male rats have a high incidence of certain types of cardiac lesions (to be reported later).

There are interesting strain differences in appearance of both coronary and general arteriosclerotic lesions. To date, selected strains were more susceptible than others. This is suggestive of the same type of situation existing in the human disease, which is known to have a predilection for certain families.

The intimal basophilia observed in some coronary arteries remains to be studied further. We hope, by histochemical means, to demonstrate whether this is calcium or mucopolysaccharide, or perhaps both existing in close association. Moon(6) has described intimal accumlation of acid mucopolysaccharide as one of the earliest changes in human arteriosclerosis.

Stress[†] would appear to be an important determinant in coronary lesions produced in rats. Three factors appear to be required: repeated breeding, unilateral nephrectomy, and administration of ACTH. It is our supposition that repeated breeding puts stress on the adrenal glands and that this stress is further heightened by administration of repeated doses of ACTH. Possibly, the unilateral nephrectomy intensifies action of ACTH. The parallelism between experimental results and those reported by Friedman *et al.*, in humans(7) is interesting and suggestive.

Summary. 1) Severe coronary arteriosclerosis has been produced in female rats (a) bred repeatedly, (b) subjected to unilateral nephrectomy, and (c) treated with ACTH. The coronary lesions in these animals resemble those seen in man. Morphologically, the coronary arteriopathy is varied, *i.e.*, intimal hyperplasia, deposition of basophilic material in intimal and medial layers (possibly acid mucopolysaccharides), elastosis, and mural swelling leading to complete obliteration of the lumen. Coronary and mural thrombi occur frequently. 2) No fat or salt is added to the diet. Serum cholesterol levels are normal. The possible role of "stress" in induction of this type of coronary artery disease has been discussed.

[†] We use the word "stress' in the broad sense as described by Selye.

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Effect of Intravenous Amino Acids on Plasma Non-Esterified Fatty Acids.* (24702)

SAMI A. HASHIM AND THEODORE B. VAN ITALLIE (Introduced by W. H. Sebrell, Jr.)
Dept. of Medicine, St. Luke's Hospital, and Inst. of Nutrition Sciences,
Columbia University, N. Y. City

Plasma levels of non-esterified fatty acids (NEFA) seem determined largely by rate at which adipose tissue releases these substances. The following operations result in decrease in concentration of circulating NEFA: Carbohydrate administration(1,2), insulin injection (1), tolbutamide administration(3), and glucagon injection(4). In contrast, increased plasma NEFA concentrations have been ob-

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served after fasting(1,5), in uncontrolled diabetes mellitus(6), after epinephrine injection (1,2,4), and after growth hormone administration(7). Fat ingestion does not appear to induce appreciable changes in level of circulating NEFA(1,2). These results suggest that rate of NEFA release from adipose cells is related to glucose utilization(8). Although effect of carbohydrate administration on NEFA metabolism has been widely studied, the effect of protein on NEFA has received little consideration. Gordon(5) mentioned that concentration of plasma NEFA decreases following ingestion, separately, of *a* L-alanine, L-glutamic acid, and L-leucine, but to a lesser degree than the decrease produced by glucose. Since parenteral, as well as oral, administration of amino acids lowers peripheral venous glucose concentration(9), an investigation was made of the effect of intravenous amino acids on concentration of plasma NEFA and blood glucose.

Methods. Eight healthy adult volunteers served as subjects. Each was studied at rest in postabsorptive state. Venous blood samples were obtained from one antecubital vein without stasis and, simultaneously, capillary blood was pipetted from finger tip, following cutaneous puncture. Fasting samples and samples following infusions of 0.9% saline solution were obtained. Six subjects received 500 ml infusion of 10% protein hydrolysate[†] over a 2-hour period, and blood samples were obtained at ½-hourly intervals during infusion. Two subjects were given 400 ml infusion of 5% L-arginine monohydrochloride[‡] in water in a 1½ hour period, and blood samples collected as described above. Capillary (arterial(10)) and venous blood glucose were measured by the Nelson modification of Somogyi procedure(11). NEFA were determined by Dole's method(1), except that constant boiling hydrochloric acid was used for standardizing the alkali used in titration. All determinations were done in duplicate. Probabilities were estimated by use of Student's test of "t"(12).

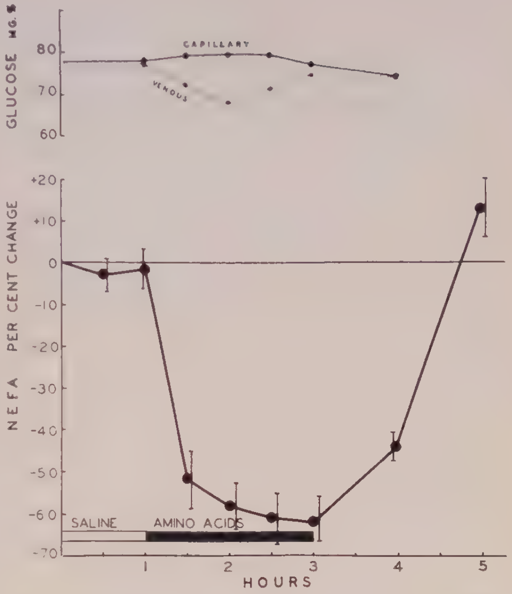

FIG. 1. Changes in mean levels of plasma NEFA (% of control) and blood glucose (capillary and venous mg %) during protein hydrolysate infusion in 6 normal subjects. Vertical bars represent one stand. error.

Results. Continued fasting for 2 hours was associated with slight elevations of NEFA levels as previously reported(1,2). Saline infusions produced no significant change in NEFA levels. In all subjects, administration of protein hydrolysate induced precipitous falls in NEFA concentration (Fig. 1). The major drop in NEFA occurred within 30 minutes after start of infusion. NEFA levels continued to fall at less rapid rate during next 90 minutes of infusion. NEFA levels tended to return toward baseline values within 60 to 90 minutes after cessation of amino acid infusion, and complete return to fasting levels occurred within 2 hours. Similar prompt decreases in NEFA concentration occurred in the 2 subjects receiving L-arginine (Fig. 2).

Concurrently with fall in NEFA levels, venous blood glucose fell significantly during the first 90 minutes of protein hydrolysate infusion. During this time capillary-venous (C-V) glucose differences were significantly increased (Table I). A progressive return to preinfusion levels of glucose C-V difference, and venous glucose concentration occurred in one-hour period following end of infusion.

[†] Amigen (glucose-free), a pancreatic hydrolysate of casein was supplied by Mead Johnson and Co.

[‡] Supplied by Cutter Labs.

TABLE I. Changes in Venous Blood Glucose and Capillary-Venous (C-V) Glucose Difference (mg %) during Infusion of 500 ml of 10% Protein Hydrolysate.

Min. after start of infusion	Mean diff. from fasting venous blood glucose	Stand. error of observed diff. between means	p	Mean C-V glucose diff.	Stand. error of diff. between means of C-V glucose	p
Fasting				− 0.9*		
30	−5.6	2.8	approx. .100	+ 7	1.2	<.005
60	−9.8	2.0	<.005	+11.5	2.7	>.005 <.010
90	−6.5	1.8	>.010 <.025	+ 8.4	1.8	approx. .005
120	−3.0*			+ 2.2*		
180	" *			0 *		

* Not significant.

During the same time, levels of NEFA tended to rise toward baseline values (Fig. 1). Increase in arteriovenous glucose difference was also observed in 2 subjects receiving arginine infusion. Values for glucose C-V differences at 30 and 60 minutes, respectively, after start of infusion were 13 and 4 mg % in the first subject, and 11 and 6 mg % in the second subject. A rise occurred in both arterial and venous glucose concentrations (Fig. 2).

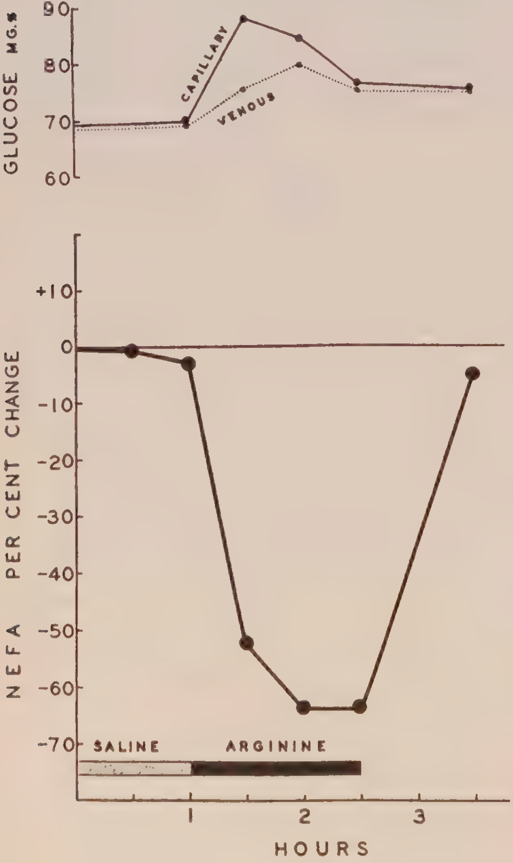

FIG. 2. Changes in mean levels of plasma NEFA (% of control) and blood glucose (capillary and venous mg %) during L-arginine monohydrochloride infusion in 2 normal subjects.

Discussion. Previous studies have suggested that rate of NEFA release from adipose tissue tends to vary inversely with concurrent rate of glucose utilization in body. Our experiments show clearly that parenteral administration of an amino acid mixture (protein hydrolysate) and administration of a single amino acid, L-arginine, were associated with prompt and dramatic fall in plasma NEFA concentration (Fig. 1, 2). This decrease in NEFA levels was sustained throughout period of infusion, but was followed by return to pre-infusion values within 90-120 minutes after infusion was ended.

The results can be considered in relation to several possible explanations. First, amino acids may directly inhibit NEFA release from adipose cells. The effect of amino acids on *in vitro* release of NEFA apparently has not been reported. A second explanation might be that administration of amino acids promotes glucose utilization, and thereby indirectly inhibits NEFA release. This might be accomplished in one of several ways: Amino acid administration might stimulate insulin release from the pancreas. Evidence of a fall in venous glucose levels and of increased glucose utilization following administration of amino acids has been reported(9,13). Since the amino acid mixture used contained an appreciable quantity of glucogenic amino acids, it is also possible that rapid conversion of these amino acids to glucose by the liver could account for the changes that occurred in NEFA.

Glucose C-V differences and venous glucose levels were measured concurrently with plasma NEFA in our study. The results show that C-V glucose differences increased significantly during administration of amino acid mixture with concomitant fall in venous glucose. Capillary glucose levels remained virtually unchanged; thus it is difficult to attribute NEFA changes to amino acid-induced hyperglycemia. Moreover, Gordon(5) reported fall in NEFA when nonglucogenic amino acid, leucine, was fed. During administration of the glucogenic amino acid, arginine, C-V glucose differences also increased, but this time, associated with a rise in capillary and venous glucose levels. This observation is in agreement with reports that response of blood glucose to amino acids differs with individual amino acids administered(14). Since blood flow through the forearm was not measured during experiments, the conclusion that rate of glucose uptake by tissues of forearm increased, must remain tentative. However, when added to observation that venous blood sugar level fell significantly during administration of amino acid mixture, these results suggest that at least some of the NEFA-lowering effects of parenterally administered amino acids occurred because of an induced increase in rate of glucose utilization.

Summary. (1) The effect of infusing an amino acid mixture (protein hydrolysate) on plasma NEFA, venous glucose level, and capillary-venous glucose difference, was studied in 6 healthy volunteer subjects. In every instance, plasma NEFA levels fell precipitously during amino acid infusion, and capillary-venous glucose differences increased concurrently. Similar results were obtained when a single amino acid, L-arginine, was infused in 2 normal subjects. (2) The results suggest that NEFA-lowering effects of parenterally administered amino acids may have been mediated entirely or in part *via* an induced increase in rate of glucose utilization.

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Absence of Intestinal Viral Flora in Infants During Epidemic of *Escherichia coli* Gastroenteritis. (24703)

VIOLA MAE YOUNG, JOEL WARREN* AND ROBERT B. LINDBERG†
Dept. of Bacteriology, Walter Reed Army Inst., Washington, D. C.

A large number of instances have been recorded in which specific strains of *Escherichia coli* have been isolated from most or all cases in outbreaks of infant diarrhea(1-6). Several

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* Present address: Chas. Pfizer Co., Terre Haute, Ind.

† Present address: USAREUR Medical Lab., Landstuhl, Germany.

TABLE I. Inoculations Carried Out with Preparations #1, 2 and 3.

Host and description	No.	Type of inoculation	No. of passages	Further inoculations
1- to 2-day-old suckling mice	1 litter	IC and IP	1 to several	None
10 to 12 g mice	6 to 8	*Idem*	*Idem*	"
Idem	*Idem*	Fed 0.25 ml via stomach tube	1	"
16 g hamsters	2	IC and IP	1	"
7- to 9-day-old embryonated eggs	6 to 8	CAS	1 to several	"
Monkey kidney Roller tubes	4 to 8	0.2 ml	3	Final harvest of 3rd passage into 1 litter suckling mice
HeLa cell Roller tubes	*Idem*	*Idem*	3	None

IC = Intracerebral inoc. IP = Intraperitoneal inoc. CAS = Allantoic sac inoc.

investigators suggested the possibility that such diarrheas are the result of interaction between the pathogenic bacterium and a virus (7-9). The following study presents an outbreak of infant diarrhea characterized by almost universal presence of *E. coli* 0-111 in fecal specimens(9) and in which intensive efforts for virus isolation gave entirely negative results.

Materials and methods. One or more fecal samples (including 6 autopsy stools) were collected from 25 infants. Two infants in this study were premature at time of onset of disease and the others were within the first 10 days after full term delivery. Time of collection ranged from day of onset to 23 days later during relapses. Two throat washings and one vomitus were also studied. Samples were frozen with dry ice and, within 4 weeks of their collection, 10% suspensions were prepared in 0.85% NaCl and centrifuged at 3,000 rpm at 4°C for ½ hour. Three inocula were prepared from each supernate. Preparation #1: a portion of supernate passed through ultrafine (UF) sintered glass filter. Preparation #2: supernate from a portion of original recentrifuged at 10,000 rpm for ½ hour at 4°C. Preparation #3: a portion of second inoculum centrifuged at 30,000 rpm for 1 hour at 4°C. The supernate was discarded and the sediment, resuspended in 0.85% NaCl with a few ml of the overlay, used for inoculation. One thousand units of penicillin and 50 μg of streptomycin/ml were added to the 2 latter preparations. Inoculations were carried out as shown in Table I. Each of the 3 preparations were inoculated into the 7 hosts listed. In addition, preparations from 7 specimens were inoculated into the yolk sac and amniotic sac as well as onto the chorioallantoic membrane of 6 to 8 embryonated hen's eggs each. Further, several specimens were inoculated intraperitoneally and intracerebrally into suckling rabbits and passaged intratesticularly through 3 to 4 adult male rabbits. Monkey kidney cultures were maintained in medium #199 plus 2% calf serum and HeLa cell cultures in a medium composed of 85% Hanks' solution, 10% chicken serum and 5% chick embryo extract ultra-filtrate.

Results. Numerous *Escherichia coli* 0-111: B_4 organisms were present in fecal samples obtained from all but 2 out of 25 patients and were also isolated from one specimen of vomitus. No *Salmonellae, Shigellae* or pathogenic protozoa were found.

All attempts to establish a transmissible virus from stool filtrates and ultracentrifugates were unsuccessful. In some instances samples were toxic for monkey kidney and HeLa cell cultures and for embryonated eggs, but repeated passages gave entirely negative results. All tissue cultures were obtained from a commercial source (Microbiological Assoc., Bethesda, Md.), and tubes from these same lots were currently being used in other laboratories for study of poliomyelitis and other agents. The same workers who carried out our studies, using the same technics and toward the end of the studies the same lots of cells, were successful in isolating Polio, Coxsacki and ECHO viruses from fecal samples collected from infants and young children from Puerto Rico and Washington, D. C.

Inability to isolate any virus from this group of severe diarrheal cases is noteworthy in view of the numbers of viruses which have since been isolated by various investigators from fecal samples of infants and young children using monkey kidney tissue cultures alone (10-14). However, Stulberg, *et al.*(15) were unable to isolate any cytopathogenic agents from a group of newborn infants with diarrhea caused by *E. coli* 0-127. Absence of viral agents from our cases, as detectable by methods employed, does not preclude the possibility of a virus actually being present, but the likelihood of any viral agent known to date causing this outbreak, either alone or in combination with *E. coli* 0-111:B_4, appears to be remote. It should be added that sera from 2 of these patients, which had been kept frozen at –20°C for the intervening period, were tested for presence of circulating antigen of *E. coli* 0-111:B_4 by means of hemagglutination-inhibition test(16) and both gave a strongly positive reaction. This result, together with negative viral findings, lends further weight to the conclusion that *Escherichia coli* 0-111:B_4 is by itself the true causative agent in some cases of highly infectious fulminating infant diarrhea.

Summary. Systematic viral isolation attempts were made from an outbreak of infant gastroenteritis in which *Escherichia coli* 0-111:B_4 was found. Treated stool samples collected from 25 infants were inoculated into monkey kidney and HeLa cell tissue cultures, embryonated eggs and a variety of young animals by various routes. No viral agent could be detected. It is concluded that this outbreak of gastroenteritis was caused by *Escherichia coli* 0-111:B_4, uncomplicated by other microbial agents.

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Effects of Total Hypophysectomy, Adenohypophysectomy, and Neurohypophysectomy on Brain Excitability. (24704)

SALVATORE DE SALVA (Introduced by N. Ercoli)
Dept. of Pharmacology and Chemotherapy, Armour Pharmaceutical Co., Kankakee, Ill.

It is generally accepted that the endocrine system exerts a definite influence on brain excitability, using the minimal electroshock seizure thresholds (EST) for determination. Age(3), ACTH(2,4,7), STH(2,6), adrenocortical steroids(2,3,7) and pitressin(6) also affect EST. The role of the pituitary gland in altering threshold values has been demonstrated(1,2,4,5,7) by measuring the changes induced by complete hypophysectomy. In view of the fact that the pituitary gland is composed of 2 lobes and that the hormones

TABLE I. Comparison of EST Changes Due to Total Hypophysectomy, Adenohypophysectomy and Neurohypophysectomy in Male Sprague-Dawley Rats in 6 Experiments.

No. rats	No. determination per rat	Age at surgery (day)	EST age (day)	EST, % difference from nonoperated controls Hx	 Antx	 Postx
35	5	36	50–59	–17.5	–23.9	–10.7
27	1	35	55	–36.7	–18.3	– 7.9
11	5	34	60–73	–24.5		
11	5	33	52–54	–20.5		
12	3	38	63	–18		
32	1	59	65	–13.5	–25.8	– 1.9

secreted by these lobes produce opposing effects on EST, the present study was undertaken. This investigation was designed to evaluate and establish how adenohypophysectomy and neurohypophysectomy carried out separately influence EST.

Method. One hundred and twenty-eight male Sprague-Dawley rats were used in 6 different experiments. Total hypophysectomy (Hx), adenohypophysectomy (Antx) and neurohypophysectomy (Postx) were carried out by the parapharyngeal approach. The accuracy of surgery was verified by gross observations of hypophyseal area and adrenal size. Electroshock seizure thresholds were determined by measuring the milliamperage required to produce facial clonia when the animals are stimulated with a 60 cps stimulator for 0.2 sec through 10,000 ohms internal resistor *via* corneal electrodes. In Exp. I, body, brain and adrenal weights were measured in the animals sacrificed 30 minutes after the last EST reading (59 days). In 3 experiments (III to V) EST measurements were made only of total hypophysectomized and nonoperated animals, while in the others, EST values were determined for Hx, Antx, Postx and non-operated controls. Average threshold effects of pituitary deficiencies are expressed (Table I) as percentage of change of operated animals in comparison to non-operated controls. Negative changes represent a lowering of thresholds or increase in excitability.

Results. Two to 4 weeks after total hypophysectomy (Exp. I to V), EST values were 17.5 to 36.7% lower than in nonoperated controls of the same age. In animals operated at 59 days of age (Exp. VI), EST was 13.5% lower than the control value. Antx rats in Exp. I, II and VI, had EST readings 18.3 to 25.8% below their controls. In contrast, EST values of Postx rats operated at a younger age (Exp. I and II) decreased by 7.9 to 10.7%. In the one group operated at a later date (Exp. VI), the threshold decreased by 1.9% only.

Body, brain and adrenal weights in Postx rats and control animals were about the same, in contrast to the considerable body and adrenal weight reduction following total and anterior hypophysectomy.

Discussion. Removal of the anterior lobe approaches the effect of total hypophysectomy on EST more than removal of the posterior lobe. In fact, in 2 out of 3 experiments, adenohypophysectomy gave a greater EST reduction than total extirpation. On the other hand, in all 3 experiments neurohypophysectomy induced a more moderate EST reduction. Total hypophysectomy induces reductions in adrenal size and EST without suppressing the zona glomerulosa.

The increase in excitability following this operation may be related to a hyposecretion of corticosteroids, since adrenalectomy gives similar effects. Maintenance of the zona glomerulosa and the fact that mineralocorticoid steroids increase EST(8) would suggest

TABLE II. Effects of Total Hypophysectomy, Adenohypophysectomy and Neurohypophysectomy on Body, Adrenal and Brain Weights.

Treatment	No. rats	Body wt, g ± S.D.	Brain wt, mg ± S.D.	Adrenal wt (2), mg ± S.D.
Hx	10	107.1 ± 5.9	1447 ± 72	12.58 ± .91
Antx	10	117.6 ± 6.3	1514 ± 39	12.89 ± 1.36
Postx	10	198.5 ±21.4	1592 ± 60	32.06 ±10.74
Intact	5	213.4 ±15.7	1588 ± 80	39.40 ± 2.86

S.D. = Stand. dev.

that these steroids are not responsible for the phenomenon noted.

On the basis of Rosenblum's(6) observation that exogenous pitressin lowers EST, one would expect that removal of an endogenous source, *i.e.*, neurohypophysectomy, should give a rise in EST. Since the opposite effect occurred, the question arises whether endogenous and exogenous pitressin influences EST by the same mechanism. The endogenous hormone may have a more complex regulatory effect, perhaps involving other endocrine factors, in contrast to the exogenous one, which may directly stimulate some portion of the central nervous system.

Summary. The effects of total hypophysectomy, adenohypophysectomy and neurohypophysectomy on EST were compared in rats. 1. Hypophysectomy and adenohypophysectomy severely lowered EST and reduced body and adrenal weight. 2. Neurohypophysectomy, although reducing EST had less effect on threshold than the other operations, without significantly altering body weight and adrenal size.

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Disturbance in Porphyrin Metabolism Caused by Feeding Diethyl 1, 4-dihydro-2, 4, 6-trimethylpyridine-3, 5-dicarboxylate. (24705)

Harvey M. Solomon* and Frank H. J. Figge†
Dept. of Anatomy, University of Maryland Medical School, Baltimore

In previous studies on fluorescence of forestomach in mice various fluorescent and nonfluorescent substances were fed to determine stainability of mucus in the forestomach. When mice that received diets containing diethyl 1, 4-dihydro-2, 4, 6-trimethylpyridine-3, 5-dicarboxylate (hereafter called "collidine derivative") were sacrificed for examination and photography of fluorescence in beam of near ultraviolet light, the gall bladder and liver appeared to be red fluorescent. Since the substance itself was intensely blue fluorescent, the red fluorescent bile and livers were thought to be a reflection of an increased porphyrin excretion. This investigation involves the study of the nature of this type of altered porphyrin metabolism. Dose relationship has been studied and types and amounts of porphyrins involved were determined.

Materials and methods. Fifty-one Strain A male mice, weighing 20 to 25 g, were fed a diet containing 2.5 g of collidine derivative/45 g ground food. Animals were sacrificed daily and liver and kidneys removed immediately for examination in beam of near ultraviolet light. One-gram samples of liver, cut so as to include the gall bladder and upper portions of biliary tree, were ground in a mortar with ethyl acetate-glacial acetic acid (4/1 v/v). Extractions were continued until red fluorescence could no longer be detected in the supernate. Total extract was fractionated according to the method of Schwartz

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* Summer Fellow, Nat. Fn. for Infantile Paralysis.

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TABLE I. Hepatic Porphyrin Concentrations in Mice Treated with Collidine Derivative. μg porphyrin/g liver.

Day on drug	No. of animals	Protoporphyrin		Coproporphyrin		Protoporphyrin/coproporphyrin
		Range	Avg	Range	Avg	
0	7			0 – trace		
1	4	48 – 160	111	trace – 15.5	9.2	11.2
2	4	58.5– 226	142.6	.36– 30	13.5	10.5
3	4	143 – 332	241	6.8 – 28.4	18.2	13.2
4	7	130 – 388	262	27.6 – 38.8	30.0	8.7
5	5	166 – 398	277.5	9.3 – 64	34.4	8.05
6	4	312 – 775	630	11.4 – 85.2	45.5	13.7
7	4	428 – 822	635	22.4 –108	55.3	11.4
8	4	550 –1056	850	43 –160	88.0	9.75
9	4	840 –1150	947	76 –177	123.5	7.7
10	4	860 –1160	976.2	128 –237	158.0	6.2
					Ratio avg	10.04

and Wikoff(1). Fluorometric analvses were carried out on aliquot samples employing a modified Lumetron Fluorometer. Additional identification of protoporphyrin fraction was carried out by study of visible absorption spectrum. The various porphyrin fractions were also analyzed by unidimensional paper chromatography technic of Erickson(2). Urinary porphyrin studies were carried out on albino male guinea pigs, fed 150 mg of collidine derivative/day in the ground food. No restrictions were placed on amount of lettuce animals received. The 24 hour urine samples, collected from animals while housed in special metabolism cages, were analyzed for porphobilinogen according to procedure of Watson and Schwartz(3) and for coproporphyrin employing 5 ml method of Schwartz, Zieve, and Watson(4). Urinary uroporphyrin was demonstrated by Al_2O_3 chromatography technic of Schwartz *et al.*(5).

Results. Data presented in Table I indicate that the oral administration of collidine derivative to mice, results in progressive increase in hepatic proto- and coproporphyrin concentrations. The ratio of protoporphyrin to coproporphyrin was approximately 10 to 1. Examination of Hematoxalin and Eosin stained sections of mouse liver from treated animals revealed presence of large numbers of dark red-brown particles in parenchymal cells, Kupfer cells, and bile ducts in portal areas. Frozen sections of treated liver, covered with a drop of glycerin and examined immediately under fluorescence microscope showed no characteristic red fluorescence. However, approximately 4 minutes after preparation, red fluorescence became evident at periphery of numerous small particles scattered throughout the unstained preparation. This fluorescence increased in intensity until it filled the microscopic field.

Examination of kidneys of mice after treatment with this agent revealed no red fluoresence until seventh day. Then, the kidney became orange-red fluorescent. When cut surfaces of sections were stained, a thin line of intense red fluorescence could be observed midway between renal cortex and pelvis. Extraction of this material in fluorescent renal tissue revealed that it consisted of large quantities of protoporphyrin and much smaller amounts of uroporphyrin.

Serum of mice treated 24 hours with collidine derivative became pink fluorescent. Characterization of porphyrins responsible for this has not been accomplished.

Studies of urinary coproporphyrin and porphobilinogen in the guinea pig treated with collidine derivative are presented in Table II. Maintenance of high urinary coproporphyrin levels appears dependent upon continued administration of the drug. When treatment with this agent was discontinued, the 24-hour urinary coproporphyrin output fell to trace quantities within 4 days. Urinary porphobilinogen, present in low concentrations on several occasions when the animal was receiving the agent does not appear to follow any characteristic excretory pattern.

TABLE II. Urinary Coproporphyrin and Porphobilinogen Excretion by Guinea Pig (740 g) Treated with Collidine Derivative, 150 mg/Day.

Day on drug	µg/24 hr Coproporphyrin	Porphobilinogen
1	0	—
2	0	—
3	Trace	—
4	49	+
5	110	—
6	158	—
7	147	Trace
8	150	"
9	138	—
10*	146	Trace
11	75.5	—
13	46.5	—
14	27.2	—
15	Trace	—

* Collidine derivative stopped.

Discussion. Abnormalities in porphyrin metabolism characterized by high levels of protoporphyrin have been discussed by Schultz and Schwartz(6). Presence of both proto- and coproporphyrin was reported by these investigators in their study of experimental chloroma. In various chloromas, protoporphyrin was approximately 20 times more concentrated than coproporphyrin. Studies of allyliso propylacetylcarbamide (Sedormid) porphyria in rabbits by Schmid and Schwartz (7) revealed increases in hepatic uro-, copro-, and protoporphyrin. Protoporphyrin was approximately 6 times more abundant than coproporphyrin in fresh livers of animals treated with this agent. Analysis of urinary porphyrins excreted, indicated a marked increase in uroporphyrin and a smaller increase in coproporphyrin. No urinary protoporphyrin could be demonstrated. Urinary porphobilinogen roughly paralleled excretion of uroporphyrin. Similar results have been reported by Case(8) for urinary porphyrin levels in rats treated with Sedormid. Illumination of viscera of rabbits treated with Sedormid(7) in beam of near ultraviolet light revealed intense red fluorescence produced by protoporphyrin in gall bladder, bile ducts, and duodenal region adjacent to orifice of common bile duct.

Though collidine derivative and Sedormid (7) are of unrelated chemical structure, the disturbance of porphyrin metabolism produced by oral administration of these agents is similar in that hepatic proto- and coproporphyrin excretion by liver, are increased in both instances. The intense red fluorescence of gall bladder, biliary tree, and duodenal region is due in each case to a marked increase in protoporphyrin.

There appear to be differences in urinary porphyrin excretion in the 2 types of chemically-induced porphyria. In animals treated with Sedormid(7) for 7 days, very large quantities of uroporphyrin and smaller amounts of coproporphyrin were present in urine. Animals receiving collidine derivative one week, excreted large amounts of coproporphyrin and smaller amounts of uroporphyrin in their urine. Urinary porphobilinogen excretion also differed. In Sedormid(7) porphyria, excretion of porphobilinogen roughly paralleled urinary uroporphyrin levels, while in porphyria induced by collidine derivative no characteristic excretory pattern for porphobilinogen could be observed.

Summary. Oral administration of collidine derivative (Diethyl 1, 4-dihydro-2, 4, 6-trimethylpyridine-3, 5-dicarboxylate) to mice produces a disturbance in porphyrin metabolism characterized by marked increases in hepatic proto- and coproporphyrin. Red fluorescence of kidneys of animals treated with this agent for 7 days is associated with great increase in renal protoporphyrin and smaller increase in renal uroporphyrin. In the guinea pig, oral administration of collidine derivative produced high levels of urinary coproporphyrin and trace amounts of urinary porphobilinogen.

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Effect of Hypothalamic Lesions on Tryptophan Peroxidase of the Liver.* (24706)

S. M. McCANN, Y. MATSUMURA, M. K. DIMICK, R. PENCHARZ AND S. LEPKOVSKY
Depts. of Poultry Husbandry, Univ. of Calif., Berkeley; of Physiology, Univ. of Pa. School of Medicine, Philadelphia; and Hooper Fn. for Medical Research, San Francisco

Independent studies show that activity of tryptophan peroxidase in the liver of rats is under partial control of the pituitary-adrenal system(1,2,3,4). Enhanced enzyme activity follows administration of "non-specific" compounds which presumably release adrenocorticotrophin (ACTH). Augmented enzyme activity is also produced by ACTH or cortical steroids(1,2,3,4). Following adrenalectomy, non-specific agents do not produce increased enzyme activity. Tryptophan, on the other hand, augments enzyme activity by a mechanism independent of adrenal cortical hormones (1). Since release of ACTH appears to be regulated, at least in part, by the hypothalamus(5,6), it appeared of interest to determine response of tryptophan peroxidase to non-specific agents, using animals with hypothalamic lesions which block secretion of ACTH. The agent chosen was histidine since it has been more thoroughly investigated than other agents(1). The results indicate that hypothalamic lesions produce a decrease in response of tryptophan peroxidase to histidine.

Methods. Adult male rats (Long-Evans) weighing 200-300 g, fed a synthetic diet(7), were used. The Krieg stereotaxic instrument was used to produce hypothalamic lesions, designed to interrupt the supraoptico-hypophysial tract in median eminence of tuber cinereum(5,6). Only rats with successful interruptions of this tract as reflected by severe diabetes insipidus with water intake in excess of 100 ml, 24 hours following induction of lesions were used. In 6 instances, location of lesions was determined by serial sections of the hypothalamic area, and the lesions lay in median eminence of the tuber cinereum. Earlier studies show that such rats subjected to acute stress give no evidence of ACTH release(5,8). Since no discernible difference in response to histidine could be detected 2, 4 and 5 days post-operatively, the combined data were pooled. Seven days intervened between hypophysectomy and histidine injection. Five hours prior to nercopsy, half the rats in respective groups received intraperitoneal (I.P.) injections of 2 mM 1-histidine-HCl; the remaining animals were uninjected and served as controls. The rats were anesthetized with Nembutal (5 mg/100 g, IP) and decapitated. Immediately afterwards the liver was removed, chilled, and a weighed portion was used in tryptophan peroxidase activity determination(1). The method used is an indirect one and depends on kynurenine production after incubation of liver homogenates with added tryptophan as substrate, activity being expressed as number of μM of kynurenine/gram dry weight liver/hour. In some cases and immediately following removal of liver, the left adrenal was removed and analyzed for ascorbic acid concentration(5). The relevant data are summarized in Tables I and II.

Results. Effect of histidine on ACTH release. It has been assumed that histidine produces its effect on tryptophan peroxidase by stimulating secretion of ACTH, which, in turn, brings about release of cortical steroids

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TABLE I. Effect of Histidine on Adrenal Ascorbic Acid Concentration of Normal Rats and Those with Hypothalamic Lesions.[*]

Normal rats		Rats with hypothalamic lesions	
Control	After histidine	Control	After histidine
337	289	415	433
303	277	458	444
305	255	425	400
408	177	401	500
398	226	445	413
252	195	515	471
357	385		
329	261		
353	296		
[†] 338 ± 15	262 ± 21	443 ± 17	444 ± 15

[*] All results expressed as mg of ascorbic acid per 100 g adrenal.

[†] Mean ± stand. error of mean.

that increase activity of tryptophan peroxidase. To test the possibiilty that histidine does release ACTH from the pituitary, ascorbic acid depletion produced by histidine injection was evaluated in normal rats and those with hypothalamic lesions. It appears that histidine does produce a small but significant ($P < .01$) decrease in adrenal ascorbic acid in the normal rat (Table I). This is interpreted to mean that histidine produces activation of the adrenal cortex by stimulating ACTH release. However, histidine appears to be far from a maximal stimulus to ACTH discharge, since it caused a decrease in adrenal ascorbic acid concentration of only 75 mg %, whereas unilateral adrenalectomy results in decrease of 190 ± 7 mg % (McCann and Fruit, unpublished observations). In contrast to normal rats, those with hypothalamic lesions failed to show any depletion of adrenal ascorbic acid after histidine injection, indicating no ACTH discharge. This would be expected from earlier studies which demonstrated that these lesions prevent ascorbic acid depletion that normally follows a variety of stresses(5,8)

Effect of hypothalamic lesions on response of tryptophan peroxidase to histidine. In normal rats histidine produced an 8-fold increase in activity of the enzyme (Table II). In contrast, rats with hypothalamic lesions were significantly less responsive ($P < .01$) as histidine caused only a 4-fold increase in enzyme activity. Preliminary experiments with hypophysectomized rats (Table II) indicate that they respond in the same way as rats with lesions, showing a significant increase in enzyme activity after histidine ($P < .05$), but a response markedly less than that of normals ($P < .01$) and indistinguishable from that of rats with hypothalamic lesions.

After hypophysectomy or hypothalamic lesions the "resting" activity of the enzyme in uninjected rats was slightly elevated above normal (Table II).

Discussion. Our results indicate that histidine does produce a mild stimulation of the pituitary-adrenal system, and also demonstrate that when this ACTH release is blocked, either by hypothalamic lesions, or by hypophysectomy, the response of tryptophan peroxidase to histidine is reduced by about 50%. To our knowledge, this is the first demonstration that hypothalamic lesions interrupting ACTH release can alter enzyme activity in the body. In other words, those alterations in enzyme concentrations which can be induced by changes in adrenocortical function

TABLE II. Effect of Histidine on Activity of Tryptophan Peroxidase in Livers of Normal Rats, Hypophysectomized Rats and Those with Hypothalamic Lesions.[*]

Normal rats		Rats with hypothalamic lesions		Hypophysectomized rats	
Control	After histidine	Control	After histidine	Control	After histidine
1.1	5.8	1.3	7.3	1.5	2.5
.7	6.3	1.6	6.0	2.4	3.8
1.0	10.6	.9	3.4	2.7	5.7
.9	7.8	1.1	3.7		5.4
.9	8.3	1.6	6.1		
1.4	6.3	1.0	8.2		
1.2	5.3	1.8	4.8		
2.0	6.3	2.2	3.5		
1.5	11.9	2.1	5.2		
1.1	10.8	1.3	4.1		
1.0	11.0	2.3	7.8		
2.0	6.0	2.1	6.8		
1.4	10.5				
1.0	13.9				
1.2	12.6				
1.2	10.9				
1.1	13.3				
[†] 1.2 ±.03	9.3 ±.7	1.6 ±.1	5.6 ±.5	2.2 ±.4	4.4 ±.8

[*] All results are expressed as μmoles of kynurenine/g liver (dry wt)/hr.

[†] Mean ± stand. error of mean.

are controlled ultimately by the hypothalamus. The results cast no light on the mechanism whereby these alterations in apparent enzyme activity occur(1,9).

The residual response to histidine found in both hypophysectomized animals and those with lesions, is of interest since it stands in contrast to the complete block of response found by Knox in adrenalectomized rats(1). This response in our experiments appears to be similar to augmented activity of the enzyme produced by histidine in cortisone-treated adrenalectomized rats(1). It thus appears that histidine can still produce an elevation in tryptophan peroxidase activity even in the presence of a constant output of cortical steroids whereas no response occurs if steroid output is zero. The role of corticoids in response to histidine, appears to fall into the category of responses which Ingle has called the permissive action of cortical steroids(10). Presumably in our experiments, the residual response to histidine must be brought about either by another regulatory system in the body, such as the sympatho-adrenomedullary system, or by a direct effect of histidine on the liver cell. Because of the multiple agents which can bring about increase in tryptophan peroxidase, this latter possibility appears to be unlikely. Another possible explanation for residual response to histidine would be that degradation of cortical steroids might be decreased under these circumstances, thereby producing a rise in blood level of steroid in the presence of a constant output of steroids as suggested by Steenberg and Ganong(11). The elevated level of steroid could then bring about the enzyme response.

Conclusions. 1. Histidine produces a small decrease in adrenal ascorbic acid concentration in normal rats, but not in rats with hypothalamic lesions interrupting the supraoptico-hypophysial tract. 2. Hypothalamic lesions or hypophysectomy produce a decrease in the response of tryptophan peroxidase to histidine but fail to block the response completely.

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A Screening Test for Malabsorption Syndromes. (24707)

A. W. HORSLEY,* JAMES A. CLIFTON, WILLIAM E. CONNOR
AND TITUS C. EVANS
Dept. of Internal Medicine and Radiation Research Lab., State University of Iowa, Iowa City

Progress in understanding of malabsorption syndromes has been hampered by lack of simple methods of diagnosis. The I^{131} labeled fat absorption test has become a valuable tool in diagnosis of these conditions but it does not fulfill all requirements of a simple reliable screening test. Plasma becomes turbid after fatty meal primarily because of increase in neutral fat or triglyceride(1). Osmon, Zinn and Whorton(2) and Turner(3) have shown that plasma lactescence fails to develop after fat ingestion in patients with chronic pancreatitis. Gardner(4) found similar results in patients with tropical sprue. We have measured changes in plasma optical density (O.D.) after fatty meal in 20 normal subjects and in 15 patients with a variety of malabsorptive defects. The data have been compared with measurements of I^{131} fat absorption, serum carotene, d-xylose excretion, and metabolic fat balance determinations.

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* Nat. Inst. Arthritis and Metabolic Disease, Trainee, 1957-58, N.I.H.

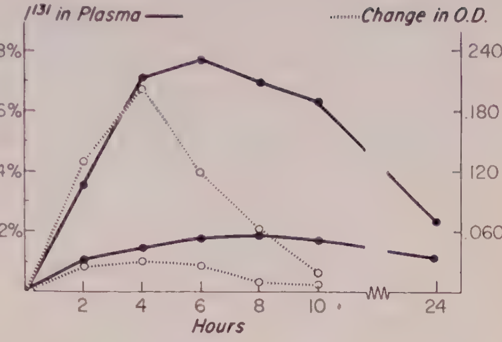

FIG. 1. Upper 2 curves show changes in plasma optical density and radioactivity in normal group, and 2 lower curves show changes in the abnormal group. Each plot represents avg of all determinations at each time interval.

Material and methods. After 12 hour fast, a homogenized meal containing 50 ml of corn oil, 240 ml of skim milk and 50 μc of I^{131} labeled triolein was given each patient. The meal has 8.2 g of protein, 14.3 g of carbohydrate, and 51.9 g of fat. Twenty drops of Lugol's solution were administered evening before the test to block I^{131} uptake by the thyroid. Eight ml of blood drawn from antecubital vein was put in heparinized tubes. Samples of blood were obtained before ingestion of test meal and every 2 hours thereafter for 10 hours. Plasma O.D. was measured in Coleman Jr. spectrophotometer at wave length of 650 mμ (12 mm light path) against water blank and expressed in optical density units. Radioactivity in 2 ml of plasma from each sample was counted in scintillation type well counter. Plasma volume was estimated from surface area and total plasma radioactivity expressed as percent of amount ingested. Seventy-two hour stool collections were counted individually and stool radioactivity was also expressed as percent of administered isotope. Amount of radioactivity in the stool was measured with gamma counter under conditions which minimize effects of variation in shape and volume of specimens. Counting was continued to give statistical reliability of at least 5%. Serum carotene was estimated by the method of Quaife, Scrimshaw, and Lowry(5), and d-xylose by that of Roe and Rice(6). Standard metabolic fat balances were measured in 5 day periods. Patients were studied for at least 2 periods. Aliquots of diets were pooled, homogenized and analyzed for fat by method similar to that of Fowweather and Anderson(7). A Goldfisch apparatus was used for extraction. Stools were pooled, homogenized and aliquots analyzed for fat. Stool fat was expressed as percent of intake. All chemical determinations were in duplicate.

Results. In the control group maximal change in plasma optical density averaged 0.260 ± 0.126 and 0.054 ± 0.052 in the abnormal group. Difference between means is statistically significant, $p<0.001$. Change in plasma optical density and rise in plasma radioactivity are shown in Fig. 1. Difference between peak values for normal and abnormals is significant at 0.1% level. Table I shows relationship between change in plasma O.D., serum carotene, urinary excretion of d-xylose, stool radioiodine, and fat balance studies in the abnormal group. For convenience patients were divided into 3 groups: primary absorptive defects, defects of absorption secondary to surgery of the G.I. tract, and miscellaneous.

In patients with primary absorptive defects the change in O.D. was less than 0.1. In 3 patients there was no change. These data agreed well with all parameters of fat absorption we studied. In patients whose malabsorption was secondary to an operation, plasma O.D. failed to rise to normal values in all but one patient, C.S. Urinary excretion of d-xylose was normal in this patient although stool radioactivity was elevated. Patients in the miscellaneous group, plasma O.D. changes were abnormal in all cases except for a single borderline value in W.B.

Table II has results before and after therapy in 2 patients with sprue and one patient

TABLE I. Comparison of Absorptive Tests in Malabsorption Syndromes in Humans.

Group		Patient	Diagnosis	Max change O.D.	Serum carotene, $\mu g/100$ ml	Urinary d-xylose, g/5 hr	Fat* balance	Stool* radioassay
I	Primary absorptive defects	M.S.	Whipples	0	22	3.5	37.2	59.9
		R.C.	Sprue	.070	21		40.5	20.8
		N.R.	"	0	12	1.4		53.9
		E.C.	"	0	0		68.9	24.9
		C.C.	"	.035	14	1.2	7.0 11.0	41.6
II	Secondary to surgery	H.S.	Subtotal gastrectomy	.059	14	4.5	28.5	63.6
		R.V.	*Idem*	.080	43			25.4
		H.M.	"	.025		.7		38.0
		B.G.	Extensive small bowel resection	0	8	1.5	82.1	91.5
		M.C.	*Idem*	.095	69	.9	12.8	46.1
		C.S.	Resection of terminal ileum	.190		6.5		29.9
III	Misc. diseases	R.B.	Fibrocystic disease	.032	20			19.1
		W.B.	Small bowel diverticula	.115 .065	43	5.5	5.5 14.7	4.1 5.0
		G.S.	Chronic pancreatitis	.014	17			18.7
		N.Y.	*Idem*	.040	8			55.7

* Stool fat expressed as % of quantity ingested.

with chronic pancreatitis. Clinical improvement was accompanied by normal rise in plasma optical density after a fatty meal.

Discussion. The fat tolerance test is a semi-quantitative measure of fat absorption. It is modified by many other factors, such as gastric emptying, rate of lymph flow, fat transport, utilization, and deposition. After ingestion of the standard fat meal all subjects in the control group had a change in plasma optical density of greater than 0.1. Therefore, we have chosen this figure as the lower limit of normal. Only 2 patients with clinical and laboratory evidence of malabsorption had rises in plasma optical density greater than 0.1. One of these, C.S., had had resection of distal portion of ileum for regional enteritis. Clinically he was malnourished and complained of explosive diarrhea, not grossly fatty. Urinary excretion of d-xylose was normal but there was an excessive amount of radioactivity in the 72 hour stool. We noted, however, that various measures of absorption in patients with postsurgery malabsorption do not agree as well as in patients with primary absorptive defects. Similar findings in serum carotene have been reported by others(8). Patient W.B. had a borderline maximal change in plasma optical density of 0.115 on one occasion, although on another the rise was only 0.065. There was similar variation in fat balance studies but stool radioactivity was normal on 2 occasions after ingestion of I^{131} labeled triolein. This patient had mild malabsorption associated with multiple jejunal diverticula. Fluctuations in fat absorption

TABLE II. Tests of Absorption before and after Therapy.

		O.D.	Serum carotene, $\mu g/100$ ml	Urinary d-xylose, g/5 hr	Stool* radioassay	Fat* balance
C.C.	Sprue in relapse	.035	14	1.2	41.6	9.0
	After gluten-free diet	.337	121	4.5	6.6	9.4
R.C.	*Idem*	.070	21		20.8	40.5
		.140	79	8.2	9.5	6.1
N.Y.	Chronic pancreatitis	.040	8		55.7	
	with pancreatin	.485			10.7	

* Stool fat expressed as % of quantity ingested.

probably reflect natural variations of the disease. All other patients with clinical and laboratory evidence of malabsorption had a rise in plasma O.D. of less than 0.1.

Summary. Our studies indicate that measurement of changes in plasma optical density after a fatty meal is a simple and reliable screening test for patients with malabsorption syndromes. It is of value in following the course of these diseases and in evaluating response to therapy.

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Effects of Hepatectomy on the Response of the Uterus to Estradiol-17-β.*

(24708)

CHARLES R. BOTTICELLI AND FREDERICK L. HISAW, JR.
(Introduced by F. L. Hisaw)
Biological Laboratories, Harvard University, Cambridge, Mass.

A peak in uterine fluid imbibition in sexually immature rats is reached within 6 hours following estrogen stimulation. This is followed by a period of cellular proliferation and gradual increase in total uterine weight(1). Hechter *et al.*(5) and Kalman(7) showed that early effects were due to a change in capillary permeability. Roberts and Szego(10-12) found that removal of two-thirds of liver greatly reduced early stimulating effect of estradiol on rat uterus. This led them to study of uterine response to estradiol at various intervals after partial hepatectomy and they observed that sensitivity to estrogen was gradually regained. They correlated restoration of estrogen activity with liver regeneration. In the present study, this relationship has been examined by using a slightly different approach and a different interpretation of data is presented.

Materials and methods. The effect of partial hepatectomy on uterine response to estradiol was studied in sexually immature rats by means of methods developed by Astwood (1). Ten animals were used in all control and experimental groups. Rats (Harvard strain) 24-25 days old and weighing 54 to 60 g were given a single subcutaneous injection of 0.1 μg estradiol 17-β immediately after 60%-70% of the liver was removed. Uteri and livers of different groups were removed at 6, 9, 12, 15, 18, 21, and 30 hours after treatment. The organs were weighed at autopsy and again after oven-drying 24 hours at 90-100°C, the difference reflecting water content. The technic of partial hepatectomy was based on those reported by Higgins and Anderson(6) and Roberts and Szego(10). The mean value and standard error are presented for key groups.

Results. On the basis of fluid imbibition, partial hepatectomy decreases normal response of uterus to estradiol as seen 6 hours after treatment (intact control, 81.8% ± 0.02%; intact treated, 87.1% ± 0.3%; hepatectomized control 81.6% ± 0.4%; hepatectomized treated, 84.2% ± 0.6%). However, when uteri were removed at various times later than 6 hours, increases in weight clearly indicate that the effect of partial hepatectomy on accumulation of water in the uterus is one of retardation rather than inhibition (Fig. 1,

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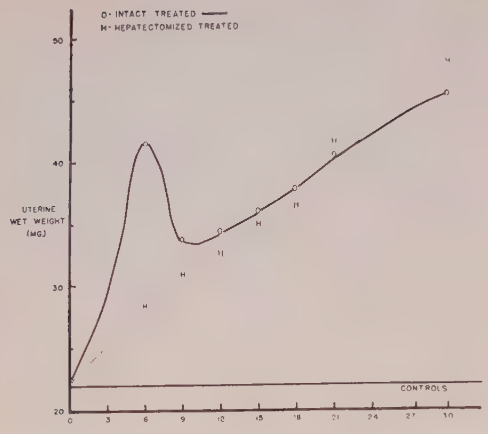
* Aided by grant from U.S.P.H.S.

3). The response reached maximum at 9-15 hours, but was not as great as in intact animals at 6 hours. It was previously reported (2) that the peak was reached between 15-18 hours, but 9-15 hours represents a more precise range as determined by larger experimental groups.

Increases in uterine dry weight over controls were used as approximate measure of uterine growth. That this is a valid criterion for growth under these conditions has been shown by correlation between dry weight and increase in total nitrogen. In our experiments, it was found that no significant difference occurred between uterine dry weights of intact treated and partially hepatectomized treated animals from approximately 9 hours to 30 hours after injection of estradiol (Fig. 2). When growth as seen at 30th hour is taken as reference point for comparison, dry weights for both groups are about the same. By this time partial regeneration of liver has occurred, to 50%-60% of normal. When 50%-60% of liver is removed at beginning of experiment, reduction of fluid imbibition and dry weights at 6 hours is approximately the same as that when 70% is removed, thus indicating that the extent by which the response during first 6 hours is decreased, is not closely correlated with amount of liver removed.

Discussion. It is difficult to explain the decreased effect of a single small dose of estradiol, when given at time of partial hepatectomy, on the basis that the liver has an important role in the action of estrogens. This is particularly true for growth phase which becomes pronounced about 9 hours after estrogen is injected. Uterine wet weight, dry weight, and per cent water beyond 15-18 hours are approximately the same for both hepatectomized and control groups, yet at 9-15 hours the liver is only 45% of normal.

Perkinson and Irving(9) report that fol-



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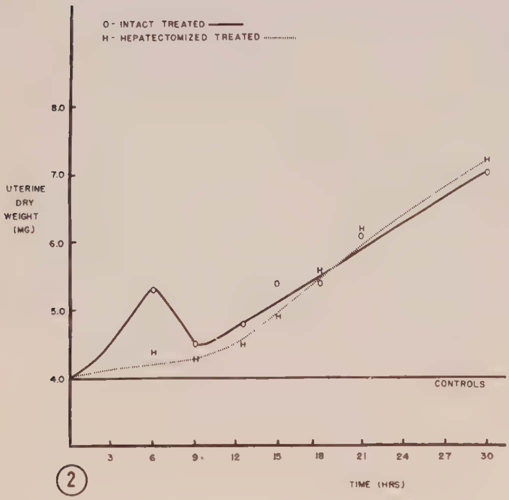

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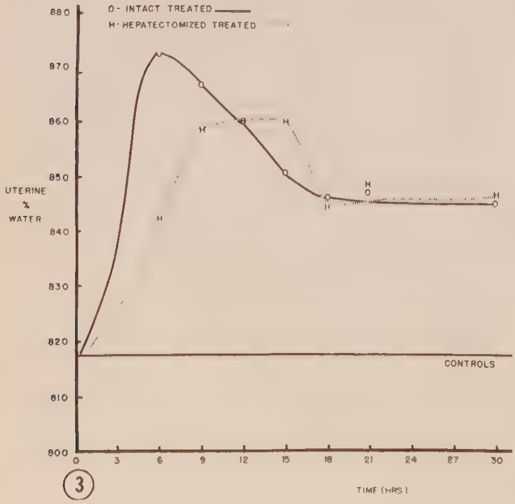

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FIG. 1. Comparison of physiological effects of estrogen on uterine wet wt in normal and hepatectomized animals treated with 0.1 μg estradiol 17-β.

FIG. 2. Physiological effects of estrogen on uterine dry wt in normal and hepatectomized animals treated with 0.1 μg of estradiol-β.

FIG. 3. Estrogen effects on % water in uterus of normal and hepatectomized animals treated with 0.1 μg of estradiol 17-β.

lowing hepatectomy, liver metabolism does not return to normal for at least 14 days although original weight may be replaced before that time. It has been found(3,6) that the most rapid rate of restoration on third day after removal of 70% of liver. Protein formation in the liver was most rapid during the first 2 days, lipid was incorporated entirely in the first day, and glycogen deposition started on second day(4). That such abnormalities following partial hepatectomy do not cause a significant difference in growth response of the uterus to estrogen gives reason for doubting that the liver is important for estrogenic action under these conditions.

The most striking effects of partial hepatectomy on uterine response to estrogen are evident over the first 9 hours of treatment. Estradiol given immediately after liver removal evoked a maximal increase in uterine water 3-6 hours later than in normal animals. Stress as a result of partial hepatectomy and consequent adrenal stimulation probably plays an important part in retardation of fluid uptake. Kramár(4) observed that rats subjected to various forms of stress showed a decrease in capillary permeability which lasted from 2-4 hours. Interestingly enough, this period is comparable to the time lag in fluid uptake in partially hepatectomized animals, and the decrease in capillary permeability may also account for the level of uterine water not being as high as the normal treated. According to Kramár(8), the immediate capillary stress response also appears in the adrenalectomized animal. This fact, plus the knowledge of effects of adrenal cortical steroids on permeability, suggests a possible explanation for the retardation of fluid imbibition by the uterus in estrogen treated hepatectomized rats.

Summary. When a single subcutaneous injection of 0.1 μg of estradiol 17-β is administered to immature rats immediately following removal of 60%-70% of liver, fluid imbibition by the uterus is retarded 3-6 hours and at maximum is somewhat less than in normal animals at 6 hours. However, partial hepatectomy does not modify growth response of the uterus to estrogen. There is no significant difference in uterine growth between control and experimental animals from approximately 15th to 30th hour following a single injection of estrogen. Since the effect of partial hepatectomy on fluid imbibition by the uterus is primarily a retardaton, and uterine growth is not changed, it seems doubtful that the liver has important influence on the action of estrogen under these conditions.

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Effect of Cold-Exposure and Fasting on Pathways of Hepatic Glucose Oxidation.* (24709)

E. J. Masoro and J. M. Felts‡
Dept. of Physiology, Tufts University School of Medicine, Boston, Mass.

Our earlier studies(1-3) have revealed that fasting rats for 24 hrs. at 0-2°C (cold-fasted rat) causes marked alterations in hepatic fatty acid metabolism. It is of great interest to know whether nutritional and environmental stress affects only fatty acid metabolism or other phases of hepatic metabolism as well. The present communication reports a study of carbohydrate metabolism in liver slices from normal and cold-fasted rats.

Methods. Adult male rats of Wistar strain were fed *ad lib.* for 10 days a diet composed of 25 g casein, 10 g fat, 51 g dextrose, 3 g salt mixture(4), 5 g cellulose, 6 g brewer's yeast, 0.04 g cod liver oil concentrate and 0.01 g α-tocopherol. After dietary stabilization, the rats were either fed the same diet *ad lib.* at room temperature (control rats) or fasted at 0-2°C for 24 hrs (cold-fasted rats). Following respective dietary and environmental treatments, the rats were killed by blow on head and the livers rapidly excised and placed in ice-cold Krebs-Henseleit bicarbonate buffer solution(5). Liver slices were prepared by a McIlwain-Buddle tissue chopper(6). The slices were collected in Petri dish containing ice-cold buffer. They were then blotted free of excess solution on filter paper, and aliquots of approximately 250 mg of tissue were weighed and transferred to incubation flasks(7). All incubations were carried out in duplicate. The main compartment of incubation flasks contained 250 mg of slices, 4.5 ml of Krebs-Henseleit bicarbonate buffer, and 0.5 ml of solution containing labeled substrate in trace amounts. The slices were incubated 3 hours at 37.5° with continuous shaking. $C^{14}O_2$ formed during incubation was recovered by the method of Chernick *et al.*(8) and the C^{14} content was determined by the $BaCO_3$ method of Entenman *et al.*(9). The C^{14} content of labeled substrate was determined by $BaCO_3$ method after oxidizing the compounds to CO_2 by the method of Katz *et al.*(10).

Results. Observations on oxidation of glucose-1-C^{14} and glucose-6-C^{14} to $C^{14}O_2$ by liver slices from control and cold-fasted rats are seen in Table I. It is not possible from these findings to calculate the precise quantitative differences in absolute amount of glucose being utilized by the 2 groups, because the large difference in hepatic glycogen content of these groups(1) makes it likely that pool sizes of the various hexose intermediates are of different orders of magnitude. However, it is clear from the data that there is a qualitative difference between livers of control and cold-fasted rats, namely that carbon 1 of glucose is forming $C^{14}O_2$ in relation to carbon 6 at a considerably faster rate in cold-fasted liver slice than in the control liver slice.

This observation might have been due only to the fact that the cold-fasted rat was accumulating the carbon 6 of glucose in an enlarged lactate pool. That this was not the case was shown by experiments with lactate-1-C^{14} and lactate-2-C^{14} (Table II) which showed that the "cold-fasted" liver was utilizing exogenous labeled lactate as rapidly as the "control" liver slice, a finding that makes it unlikely that carbon 6 is being trapped in an enlarged lactate pool of the "cold-fasted" liver. The data, therefore, indicate that relatively more metabolized glucose is being utilized *via* the hexose monophosphate pathway in the case of "cold-fasted" liver than in the "control" liver.

Discussion. Metabolism of fatty acids by liver slices from cold-fasted rats is quite different from that of liver slices from control rats(1-3). Short-chain fatty acids are oxi-

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‡ Present address: Research Specialties Co., 200 So. Garrard Blvd., Richmond, California.

TABLE I. Glucose Oxidation in Liver Slices from Cold-Fasted and Control Rats.*

Environment and nutrition	% glucose-1-C^{14}	% glucose-6-C^{14}	$\frac{C^{14}O_2 \text{ from glucose-6-}C^{14}}{C^{14}O_2 \text{ from glucose-1-}C^{14}}$
	recovered in CO_2		
Fed at 25°C	3.0 ± .35†	2.0 ± .37	.64 ± .076
Fasted at 0-2°C	3.9 ± .58	1.3 ± .24	.33 ± .016

* Values represent avg for 5 rats, 4 aliquots of liver slices incubated from each rat, 2 with glucose-1-C^{14} and 2 with glucose-6-C^{14}. The ratio $\frac{C^{14}O_2 \text{ from glucose-6-}C^{14}}{C^{14}O_2 \text{ from glucose-1-}C^{14}}$ was determined for each rat and avg ratio reported for each group of rats.

† Stand. dev. of mean.

dized at a much slower rate in liver slices of the cold-fasted rat, a defect that can be repaired by adding glucose to the incubation medium. Long-chain fatty acids are oxidized at normal rates by the "cold-fasted" liver but on addition of glucose to the incubation medium the rate of long-chain fatty acid oxidation becomes much faster than that of normal liver. These results point to the important role of carbohydrate metabolism in relation to fat oxidation.

In the present report it is shown that the combined stress of cold-exposure and fasting not only affects fatty acid metabolism but carbohydrate metabolism as well. As stated above, the evidence suggests that carbohydrates are diverted towards the hexose monophosphate pathway in the liver of cold-fasted rat. This is a very surprising finding when one considers that fatty acid synthesis is almost non-existent in these livers(1,2). Tepperman and Tepperman(11) reviewed the literature and presented new evidence that implicates TPNH production *via* the hexose monophosphate pathway as a strong stimulator of fatty acid synthesis. However, no lipogenesis ocurs in the "cold-fasted" liver even under conditions where an abundance of carbohydrate is available and when, according to our report, a large part of carbohydrate metabolism is occurring *via* the hexose monophosphate pathway. There must, then, be a dearth of other factors necessary for lipogenesis in the "cold-fasted" liver.

TABLE II. Lactate Oxidation in Liver Slices from Cold-Fasted and Control Rats.

Environment and nutrition	% lactate-1-C^{14} recovered in CO_2	% lactate-2-C^{14} recovered in CO_2
Fed at 25°C	56	22
Idem	56	27
"	51	24
"	56	26
Fasted at 0-2°C	66	25
Idem	61	23

Summary. Metabolism of glucose-1-C^{14} and glucose-6-C^{14} was studied in liver slices from control rats (fed at 25°C) and cold-fasted rats (fasted 24 hours at 0-2°C). Evidence is presented that suggests that carbohydrate metabolism is diverted towards the hexose monophosphate pathway in the liver of cold-fasted rats. The depressed hepatic fatty acid synthesis in the cold-fasted rat is discussed in the light of this new evidence on carbohydrate metabolism.

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Prevention of Steroid Induced Ulcers with an Anticholinergic Drug. (24710)

ANDRÉ ROBERT AND JAMES E. NEZAMIS
Dept. of Endocrinology, The Upjohn Co., Kalamazoo, Mich.

Since development of peptic ulcer is one of the clinical side effects of corticoid therapy, an assay was recently developed to better study the pathogenesis of this type of ulcer. In this assay, ulcers are produced in the glandular portion of rat stomach in 4 days of intensive corticoid treatment(1). The results are expressed in terms of "ulcer index." In the present experiment, an anticholinergic drug, methscopolamine bromide,* was administered together with an ulcerogenic dose of Δ^1-cortisol (Δ^1-COL) in order to assess the rôle of vagal influences in the formation of this lesion.

Materials and methods. Eighty female Sprague-Dawley rats of average body weight of 155 g were divided into 8 groups (Table I). The animals were placed in individual cages and, beginning at 9 a.m., food was withheld for 4 days. During fasting period, they were treated with compounds listed in Table I, in a volume of 0.2 cc subcutaneously at following doses: Methscopolamine: either 1, 3 or 5 mg/kg of body weight twice daily (this corresponded to 80, 240 and 400 μg/injection respectively); Δ^1-COL: 5 mg once a day. Group 1 received 0.2 cc of CMC (carboxymethylcellulose, polysorbate 80, propylparaben and water), which was the vehicle for both methscopolamine and Δ^1-COL, subcutaneously, once a day. In addition, all animals were given a mixture of 5000 u of Penicillin G crystalline and 4 mg of dihydrostreptomycin sulfate subcutaneously twice a day. The antibiotics were mixed with methscopolamine in groups receiving anticholinergic. In other groups (Groups 1 and 5) they were given in separate injections of 0.2 cc. Animals were killed with chloroform after 4 days, and body weights recorded. Stomachs were dissected out, opened along lesser curvature and examined under a binocular magnifier for presence of ulceration.

Results. Table I summarizes the data obtained for each group.

Forestomach ulcers. A 4-day period of fasting induced formation of edema and ulcerations in this nonglandular portion in 40% of control animals, with an average of 4.1 ulcers/rat. Both methscopolamine and Δ^1-COL, either alone or in combination, completely prevented appearance of these ulcers.

Glandular ulcers. Control animals (Group 1) showed an incidence of glandular ulcers of 20%, and these were very mild. In the authors' experience, such low incidence and severity can be considered negligible; most often, controls do not show any glandular ulcers. Δ^1-COL produced, as usual, a high incidence of numerous and severe ulcers. When methscopolamine was given together with Δ^1-COL, it prevented formation of glandular ulcers proportionately to dose. In fact, with the highest dose of 5 mg/kg, the "ulcer index" was identical to that of the controls.

Mucus. Although the amount of mucus was not measured, it was obvious that with methscopolamine alone the gastric surface was covered with a thicker layer than in control animals. On the other hand, no mucus was visible on mucosa of animals receiving Δ^1-COL even when methscopolamine was also administered.

Discussion. The present experiment confirms the observation that glucocorticoids prevent appearance of fasting ulcers in the forestomach(1). It was previously shown that forestomach ulcers that develop in the Shay rat (pylorus ligation in fasted animal) are also inhibited by glucocorticoid treatment(2). Methscopolamine has been reported to prevent Shay ulcers as well as fasting ulcers in the forestomach(3).

Our results support the clinical findings that anticholinergic drugs are of value in treatment of peptic ulcer. Furthermore, they can specifically prevent development of ulcers

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* Pamine, Registered trademark, Upjohn Co., Kalamazoo, Mich.

TABLE I. Effect of Methscopolamine Bromide (Methsc) on Steroid Induced Ulcers (Glandular portion) and on Fasting Ulcers (Forestomach).

Group	1	2	3	4	5	6	7	8
						Δ^1-COL		
		Methsc						
Treatment	CMC	1 mg/kg	3 mg/kg	5 mg/kg	Δ^1-COL	Methsc, 1 mg/kg	Methsc, 3 mg/kg	Methsc, 5 mg/kg
Initial body wt	155	155	156	155	155	156	155	156
Final " "	111	110	110	109	102	101	100	101
Difference	–44	–45	–46	–46	–53	–55	–55	–55
Forestomach								
Edema, %	40	0	0	0	0	0	0	0
Ulcers, %	40	0	0	0	0	0	0	0
No./rat	4.1							
Glandular ulcers								
%	20	0	0	0	100	100	90	20
Severity (+)	.2				1.8	1.1	.6	.2
No./rat	.4				6.8	4.1	1.6	.4
Ulcer index*	2.6	0	0	0	18.6	15.2	11.2	2.6

* "Ulcer index" for glandular portion of stomach is determined by calculating the sum of: incidence in % divided by 10, severity (in pluses) and No. of ulcers/rat. A maximum "ulcer index" is around 20.

due to corticoid therapy. In this same connection, Carbone *et al.*(4) made the interesting observation that while prednisone increased both gastric pepsin and urinary uropepsin in normal subjects, addition of an anticholinergic drug nullified these effects of the steroids. According to our results, there is also a possibility that part of the protection afforded by methscopolamine was due to stimulation of mucus secretion.

Summary. Ulcers were produced in glandular portion of stomach of fasting rats with Δ^1-cortisol (Δ^1-COL). When methscopolamine bromide, an anticholinergic, was administered, these steroid induced ulcers were prevented proportionately to dose. Methscopolamine, given alone, increased the amount of visible mucus on mucosal surface. Methscopolamine would protect by counteracting some effects of corticoids on gastric secretion and perhaps also by stimulating mucus formation.

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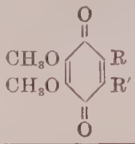
Coenzyme Q. IV. Coenzyme Activity of 6-Isoprenoid and Other Derivatives of 2,3-Dimethoxy-5-Methylbenzoquinone. (24711)

FREDERICK L. CRANE, C. H. SHUNK, F. M. ROBINSON, AND K. FOLKERS
(Introduced by Hans Molitor)
Inst. for Enzyme Research, University of Wisconsin, Madison, and
Merck Sharp & Dohme Research Labs., Rahway, N. J.

Discovery(1) and chemical structures(2) of the coenzyme Q group and additional characterization of ubiquinone(3) have been described recently. Synthesis of 6-(3′-methyl-2′-butenyl)-, (IV), 6-geranyl-, (V) and 6-farnesyl-, (VI), derivatives of 2,3-dimethoxy-5-methylbenzoquinone has been reported(4). These 3 compounds are lower isoprenoid analogs of the coenzyme Q group and may be considered as coenzyme Q_1, Q_2, and Q_3, respec-

TABLE I. Coenzyme Q Assay on 2,3-Dimethoxybenzoquinone and Certain Derivatives.

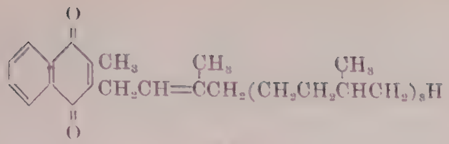


	Compound	Sample, mg	Specific activity succinoxidase	△S.A./μM
I	$R = R' = -H$	.025	.00	.0
II	$R = -CH_3$	”	.00	.0
	$R' = -H$	.05	.00	.0
III	$R = R' = -CH_3$	.025	.06	.47
		.05	.08	.31
IV	$R = -CH_3$	.025	.11	1.0
	$R' = -CH_2CH = \overset{CH_3}{\overset{\vert}{C}}CH_3$	.049	.16	.73
	(coenzyme Q_1)			
V	$R = -CH_3$	.0094	.19	5.9
	$R' = (-CH_2CH = \overset{CH_3}{\overset{\vert}{C}} - CH_2)_2H$	.047	.28	
	(coenzyme Q_2)			
VI	$R = -CH_3$	.011	.14	4.8
	$R' = (-CH_2CH = \overset{CH_3}{\overset{\vert}{C}} - CH_2)_3H$	.053	.29	
	(coenzyme Q_3)			
VII	$R = -CH_3$	.025	.19	3.5
	$R' = -CH_2CH = \overset{CH_3}{\overset{\vert}{C}}-CH_2(CH_2CH_2\overset{CH_3}{\overset{\vert}{C}}HCH_2)_3H$	(–phytyl)		
	(hexahydrocoenzyme Q_4)	.05	.25	
VIII	$R = -CH_3$	.025	.15	5.0
	$R' = (CH_2CH = \overset{CH_3}{\overset{\vert}{C}} - CH_2)_{10}H$	.05	.25	
	(coenzyme Q_{10})	.25	.28	
	None		0	
	Mixture of II and Q_{10}	.025 ”	0	
	Mixture of III and Q_{10}	” ”	.10	

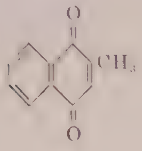
tively. The 6-phytyl derivative (VII) of 2,3-dimethoxyl-5-methylbenzoquinone (hexahydrocoenzyme Q_4) was also synthesized(4). VII and Vit. K_1 have the same isoprenoid substituent. These 4 new isoprenoid compounds and the unsubstituted, 5-methyl-, and 5,6-dimethyl- derivatives of 2,3-dimethoxybenzoquinone have been tested for activity as coenzymes in the succinoxidase system; the data are in Table I.

Methods. The electron transport particle (5) was extracted at room temperature with 4 volumes of isooctane by rapid shaking for 1 hour on a reciprocal machine in presence of pH 5.5 potassium acetate buffer (0.4 M) and versene (0.002 M). Succinoxidase activity of the extracted particle was assayed manometrically as described previously(6) with mitochondrial phospholipid as supplement (.03 mg/flask). The extraction procedure removes coenzyme Q completely but does not remove other lipid components required for reactivation of more extensively extracted particles. The assay system used to collect data here reported did not contain the neutral lipid (NL II)(5) as supplement. Oxidation rates in the assay system were linear over a 20 minute period.

Results. (Data in Table I) 2,3-Dimethoxybenzoquinone (I) showed no activity. 2,3-Dimethoxy-5-methylbenzoquinone (II) also showed no activity, and when tested in pres-



IX



X

ence of coenzyme Q_{10} completely inhibited oxidation. This 5-methyl derivative (II) has the same structural relationship to 2,3-dimethoxy-5-methyl-6-phytylbenzoquinone (VII) (and essentially to Q_{10} also) as menadione (X) has to Vit. K_1 (IX).

2,3-Dimethoxy - 5,6-dimethylbenzoquinone (III) elicited very low activity and did not restore the full rate, even at higher concentrations. When this 5,6-dimethyl derivative (III) was tested in presence of coenzyme Q_{10}, there was inhibition.

Coenzyme Q_1 (IV) showed low activity and did not restore full rate. Coenzymes Q_2 (V) and Q_3 (VI) showed the same specific activity and restored the full rate. The hexahydrocoenzyme Q_4 (VII) showed full activity, and is of particular interest; it is an isoprenoid analog of intermediate chain length which is readily synthesized, because of availability of phytol. Furthermore, the 6-phytyl derivative possesses a 2′-double bond adjacent to the quinoid nucleus which may contribute to maximal electron functionality of the nucleus and coenzymatic activity.

Importance of the 6-isoprenoid substituent in coenzyme Q_{10} (VIII) to its lipid characteristics is more evident if one considers that the molecule has a 9-carbon quinoid nucleus attached to a 50-carbon hydrocarbon side chain. Although the coenzymatic activity of Q_6, Q_7, Q_8 and Q_9(1) and some of the synthetic analogs described herein show that considerable variation of the 9-carbon/50-carbon ratio is possible with retention of activity, it is evident that excessive reduction of the 50-carbon side chain destroys the dominant lipid characteristics and coenzymatic activity of the molecule.

Studies on structure-activity relationships in the coenzyme Q group are being extended.

Summary. 2,3 - Dimethoxybenzoquinone and 2,3 - dimethoxy - 5-methylbenzoquinone showed no coenzymatic activity in a succinoxidase system. 2,3-Dimethoxy-5,6-dimethylbenzoquinone and coenzyme Q_1 showed low activity. Coenzyme Q_2, Q_3 and hexahydrocoenzyme Q_4 showed activity comparable to that of coenzyme Q_{10}.

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Plaque Formation by Infectious Canine Hepatitis (ICH) Virus. (24712)

S. Levine, V. J. Cabasso, J. M. Avampato and M. R. Stebbins
(Introduced by H. R. Cox)
Viral and Rickettsial Research Section, Lederle Labs., American Cyanamid Co., Pearl River, N. Y.

It is generally agreed that ability of an animal virus to form plaques on a susceptible cell monolayer parallels its ability to be cytopathogenic for this cell. Although several years have elapsed since infectious canine hepatitis (ICH) virus was shown to multiply in dog-kidney tissue culture and to cause characteristic cytopathic changes(1), plaque production with this virus has not been reported. Attempts have been made without success. This communication describes production of plaques by ICH virus derived from either dog or swine kidney cultures.

Materials and methods. The *ICH virus* used was isolated in our laboratories as previously described(2). Two preparations were employed for plaque assay: one representing 37th passage of virus in dog-kidney tissue culture, and the other 30 transfers in swine kidney(3). *Cultivation and titration.* Dog and swine-kidney cultures were prepared by method of Dulbecco and Vogt(4) in either stationary tubes (1 ml) or Povitský bottles (100 ml). Dog kidney cells were also grown in 50 mm Petri dishes (5 ml). Tubes were used for serial transfer of the virus, and bottles for preparation of large pools. Growth medium consisted of Earle's basal solution(5) containing lactalbumin hydrolysate and 15 or 20% calf serum. Just before inoculation, tubes and bottles were renewed with mixture 199(6), supplemented for dog-kidney tubes, with 2% horse serum. Virus titrations were carried out in dog kidney tubes and were terminated after 7 days at 37°C. Fifty % endpoint tissue culture doses (TCD_{50}) titers were calculated by Reed and Muench formula(7). *ICH immune serum.* This was prepared in foxes as described earlier(1). Immune and normal sera were inactivated at 56°C for 30 minutes before use in the neutralization test. *Serum neutralization.* To one set of serial 10-fold dilutions of virus in glucosol(8) were added equal volumes of glucosol; a second set received equal volumes of 1:16 dilution of normal dog serum, and a third set equal volumes of 1:16 dilution of ICH immune fox serum. After incubation in a 37°C water bath for 1 hour, each serum-virus mixture was inoculated into 3 culture tubes at 0.1 ml/tube. These were incubated at 37°C and observed for 7 days. TCD_{50} titers were calculated as indicated above.

Results. Plaque production. Plates of primary passage dog-kidney monolayers were washed once with 3 ml phosphate buffer saline solution (PBS)(9). Appropriate dilutions of ICH virus of either dog- or swine-kidney origin, made in Eagle's medium(10) fortified with 1% calf serum, were added to the monolayers in 1 ml amounts. The plates were then incubated at 37°C for 3 hours to permit virus attachment, at which time they were overlayed with 7 ml of a medium consisting of 5% calf serum, 1% agar and 1:40,000 neutral red in Eagle's medium. Incubation was continued at 37°C in an atmosphere of 5% CO_2 in air. The plaques appear sometime between fifth and tenth day (Fig. 1). Under the same conditions, equally successful results were obtained with second passage dog kidney cells.

Reproducibility and precision of assays. To determine validity of the plaque method for assay of ICH virus, several virus stocks of either dog- or swine-kidney origin were plated on different days, using 2 or 3 dilutions/assay. Results are given in Table I. A linear relationship was obtained between number of plaques and viral concentration, over a relatively wide range of dilutions. This suggests that a plaque can arise from infection with a single virus particle. Reproducibility and precision obtained in these experiments seem also to be as good as those seen with other animal viruses(4,11,12,13) and bacteriophage(14).

Plaque identification. The plaque producing virus was identified as ICH by tube and plaque-assay neutralization with known specific serum.

Comparison of tube and plate assays. Com-

FIG. 1. Plaques of ICH showing effects of dilution.

TABLE I. Representative Experiments Demonstrating Reproducibility of Plaque Count and Its Response to Dilution.*

Virus stock	Assay No.	Dilution	Plaque count	Titer of stock
A	1	$1/10^5$	33,38	3.6×10^6
		$1/10^6$	5,1	3.0 × "
	2	$1/10^5$	30,32	3.1 × "
		$1/10^6$	3,4	3.5 × "
B	1	$1/10^5$	48,32	4.0 × "
		$1/10^6$	3,3	3.0 × "
	2	$1/10^5$	69,57,49	5.8 × "
		$1/10^6$	2,3,4	3.0 × "
C	1	$2/10^4$	51,46,69	2.7 × "
		$5/10^5$	6,16,20	2.8 × "
		$1/10^6$	3,7,0	3.0 × "
D	1	$5/10^5$	59,78,82	1.5×10^7
		$1/10^6$	17,22,12	1.7 × "

* Experiments with same stocks were done on different days.

parative titrations of various virus stocks were carried out by plaque assay and by standard dog-kidney tube method. Results are shown in Table II. Under conditions of these experiments, it appears that plaque assay is more sensitive than tube method.

Discussion. Under appropriate conditions ICH virus of either dog- or swine-kidney culture origin can form plaques on monolayers of 1st or 2nd passage dog kidney cells. Moreover, our data justify the use of the plaque method for assay of the virus. It also supports the concept that the ICH plaque, like plaques formed by other animal viruses studied(11,12,13), can be initiated by a single infective particle. The seeming discrepancy between titers obtained by tube and plate methods, may be due to the fact that tube titrations were routinely terminated on 7th day.

It has not yet been possible to determine relative plating efficiency of swine-kidney and dog-kidney grown virus on both dog- and

TABLE II. Comparative Titrations of ICH Virus Stocks by Plaque Assay and by Standard Dog-Kidney Tube Method.

Virus stock	Dog-kidney tube assay: Infective particles/ml*	Plaque titer: PFP/ml†
1	2×10^7	2.9×10^8
2	2×10^6	5.8×10^6
3	2 × "	1.4×10^7

* One ID_{50} corresponds, on the average, to 0.7 tube infective particle.

† PFP = plaque forming particle.

swine-kidney monolayers because of the difficulty in keeping primary swine cultures for the long time needed for plaque production. However, work on this problem is being continued.

Summary. Plaque formation by infectious canine hepatitis virus was demonstrated on dog kidney monolayers with both dog and swine kidney grown virus. A plaque-derived virus clone was identified as ICH by stationary tube neutralization test as well as by plaque assay. Under conditions of the study, assay by plaque plates appears to be a more sensitive titration method than by stationary tissue culture tubes.

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Experimental Aminonucleoside Nephrosis (II): Effect of Adrenalectomy on Fluid Retention of Aminonucleoside Nephrosis.* (24713)

D. Das Gupta, N. Kalant and C. J. P. Giroud† (Introduced by J. S. L. Browne)
Dept. of Investigative Medicine, McGill University, Montreal

In young immature rats, aminonucleoside nephrosis is characterized by proteinuria, hypoproteinemia, hypercholesterolemia, increased rate of secretion of aldosterone, and fluid retention of which 50% is in the form of ascites(1-3). To appraise the relationship of hyperaldosteronism to fluid retention, the ascites produced by aminonucleoside nephrosis was measured in adrenalectomized rats receiving maintenance doses of corticosterone and maintenance or increased doses of either desoxycorticosterone or aldosterone. The results strongly suggest that in aminonucleoside nephrosis an increased rate of production of aldosterone is one, but not necessarily the only, causative factor in gross fluid retention.

Methods. Immature rats of Long-Evans strain, weighing 90-100 g, were used and fed a diet of Purina Fox Chow. Following adrenalectomy the animals were maintained on 1% sodium chloride in drinking water. Four days after operation the saline solution was replaced by tap water and maintenance therapy with aldosterone and corticosterone was started. After 4-5 days of maintenance therapy, nephrosis was induced by single daily subcutaneous injections of 6-dimethyl-aminopurine 3-amino-d-ribose at dose of 1.75 mg in 0.2 ml of water/rat/day for 8 consecutive days. Six days following last injection of the nephrotoxic drug, the animals were killed by bleeding from inferior vena cava. Development of nephrosis was established on the basis

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† Holder of Canadian Life Insurance Officer's Assn. Fellowship.

TABLE I. Development of Aminonucleoside Nephrosis in Adrenalectomized Rats.

	Total serum proteins, g %	Serum albumin, g %	Serum cholesterol, mg %	Ascites, ml/kg of body wt
Intact controls (9)	5.4 ± .7*	3.4 ± .1	63 ± 9	0
Intact nephrotics (10)	3.7 ± .7	1.7 ± .6	394 ±107	69 ± 21
Adrenalectomized—Series A				
Controls (8)	5.2 ± .6	3.6 ± .7	51 ± 7	0
Nephrotics (10)	4.1 ±1.2	1.9 ± .6	200 ± 59	0
Adrenalectomized—Series B				
Controls (8)	4.8 ± .3	3.5 ± .3	85 ± 14	0
Nephrotics (11)	4.1 ± .7	1.9 ± .6	250 ± 43	60 ± 20
Adrenalectomized—Series C				
Controls (9)	5.2 ± .3	3.3 ± .2	57 ± 14	0
Nephrotics (5)	4.1 ± .5	1.9 ± .4	273 ± 32	62 ± 37

* Stand. dev. of mean.

Numbers in parentheses indicate No. of rats. Rats of Series A were maintained on aldosterone and corticosterone. Series B and C received high doses of desoxycorticosterone and of aldosterone respectively.

of hypoalbuminemia(4), hypercholesterolemia (5), and characteristic changes in electrophoretic pattern of plasma proteins. Just before exsanguination volume of ascites was carefully measured by withdrawal into a syringe.

Results. Six days after last of 8 injections of aminonucleoside, intact nephrotic animals, as compared to normal rats, present hypoproteinemia, mostly accounted for by decrease of serum albumin, a marked degree of hypercholesterolemia and mean volume of ascitic fluid of 69 ± 21 (S.D.) ml/kg of body weight (Table I).

Maintenance therapy for adrenalectomized rats consisted of aldosterone and corticosterone in daily doses of 1.5 γ and 220 γ/100 g of body weight, respectively. Although these values were established by taking into consideration secretion rate of these hormones in adrenal vein blood of normal rats(3), it is doubtful that they represent more than an approximation of daily output of aldosterone and corticosterone by adrenals of intact rats. The total serum proteins, albumin, and cholesterol of adrenalectomized control rats receiving this maintenance therapy did not differ from those of intact rats.

Adrenalectomized rats receiving this therapy and injected with aminonucleoside presented a degree of hypoalbuminemia and hypercholesterolemia comparable to that found in intact nephrotic rats. There was however, a striking absence of ascites (Table I).

Two groups of adrenalectomized rats (Series B and C of Table I) were maintained on replacement doses of aldosterone and corticosterone for the period preceding injections of aminonucleoside. From this time until end of experiment, these 2 series of animals received respectively desoxycorticosterone acetate at dose of 400 γ and aldosterone at 16 γ/100 g body weight/rat/day. These dosages were chosen to simulate increased rate of production of aldosterone previously demonstrated in adrenal vein blood of nephrotic animals(3). The dose of corticosterone was unchanged. After injection of aminonucleoside, animals in both series presented a degree of hypoalbuminemia and hypercholesterolemia comparable both to that of intact nephrotic rats and to that of adrenalectomized nephrotic rats receiving maintenance doses of adrenal hormones; in contrast to the latter, a severe state of fluid retention was observed, as shown by volume of ascites virtually identical with that of intact nephrotic rats. Adrenalectomized, nonnephrotic rats, receiving same dosage of corticosterone and either desoxycorticosterone acetate or aldosterone as the nephrotic animals in Series B and C, presented levels of total serum proteins, serum albumin and cholesterol strictly comparable to those of intact controls and did not develop fluid retention.

Discussion. Singer(2) first demonstrated that aminonucleoside nephrosis in rats is accompanied by increased rate of production of aldosterone. Further studies of this experimental syndrome have indicated that 4-androsten-3-one 17-spirolactone, an inhibitor of mineralocorticoids at kidney tubule level, promotes loss of ascites in aminonucleoside nephrotic rats without affecting their high rate of secretion of aldosterone (unpublished). By demonstrating that on maintenance doses of aldosterone, adrenalectomized nephrotic rats do not accumulate ascites whereas on higher doses of either aldosterone or desoxycorticosterone a state of fluid retention comparable to that observed in intact nephrotic rats is induced, the present data strongly suggest that hyperaldosteronism of aminonucleoside nephrosis is responsible for the presence of severe fluid retention. The same dosage of mineralocorticoids is without apparent noxious effects in adrenalectomized control rats.

Surtshin(6) reported severe fluid retention in adrenalectomized aminonucleoside nephrotic rats maintained on 1% NaCl in drinking water, but without any supportive therapy with adrenal hormones. We have confirmed this observation; this, together with results described above, suggests that fluid retention of aminonucleoside nephrosis is secondary to a decreased ability to excrete sodium. Hyperaldosteronism is one factor contributing to this disability.

Summary. Gross fluid retention and hyperaldosteronism are 2 characteristic features of aminonucleoside nephrosis in rats. Adrenalectomized aminonucleoside nephrotic rats receiving maintenance doses of aldosterone and corticosterone do not present fluid retention, whereas they do upon administration of aldosterone at doses equivalent to amount of this hormone secreted by adrenals of intact nephrotic animals.

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Cardiovascular and Respiratory Effects of Lethal Doses of Histamine and Compound 48/80 in Rabbits.* (24714)

CHARLES A. PAPACOSTAS† AND EARL R. LOEW
Depts. of Physiology, Boston University School of Medicine, Boston, Mass., and Pharmacology, Temple University School of Medicine, Philadelphia, Pa.

Compound 48/80 has been reported a potent liberator of endogenous histamine, capable of producing the main features of histamine and anaphylactic shock in several species(1,2). It is probable that 48/80 exerts pharmacologic and toxic effects which are unrelated to histamine released from tissues. Experiments have been made, using artificial respiration, to distinguish the effects of lethal doses of 48/80 and histamine on respiratory and cardiovascular systems of the rabbit, in which species 48/80 does not induce marked hypotension(2).

Methods. Albino rabbits of both sexes weighing from 3.0 to 4.6 kg were anesthetized with urethane (25% aqueous solution intravenously; 1.2 g/kg). The trachea was intubated with T-shaped glass cannula and respiration recorded semi-quantitatively on kymograph by connecting one of the outlets of the cannula to a recording bromoform manometer or to a rubber tambour. Femoral or carotid mean arterial pressure was recorded

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† Present address: Dept. of Pharmacology, Temple Univ. School of Med., Philadelphia, Pa.

kymographically with a mercury manometer. Artificial respiration using room air was administered by means of a standard respirator at 24 strokes/minute. Volume input was regulated to produce moderate chest expansion. All injections were made into marginal ear vein during 10 seconds. The volumes injected ranged from 0.4 to 1.5 ml. *Compound 48/80, Group 1.* Twenty-six animals received 2.25 mg/kg representing 1½ times the LD_{50} (1.5 mg/kg) predetermined in the unanesthetized animal. Artificial respiration was instituted one minute after respiratory arrest, or when blood pressure fell below 70 mm Hg even though feeble respiration was still present. At 10 minute intervals for approximately an hour, artificial respiration was interrupted for one to 2 minutes to determine whether animals would respire spontaneously. The procedure was then supplemented in some animals with use of certain analeptic drugs each administered to 3 different animals in the following manner: pentylenetetrazol 10 to 20 mg/kg, amphetamine sulfate 3.0 mg/kg and nikethamide 50 mg/kg injected intravenously in a volume of 2.0 ml over a 2 minute period. Upon completion of infusion, artificial respiration was again stopped for brief interval. In the absence of any breathing, artificial respiration was resumed. When possible a second injection was made 20-30 minutes after the first. *Group 2.* Responses to 48/80 in animals of Group 1 did not suggest that histamine liberation from tissues played an important causative role (see Results). Therefore 9 additional rabbits were injected with 3 times the LD_{50} of 48/80 (4.5 mg/kg) to determine whether responses similar to those induced with histamine could be elicited. In these animals, artificial respiration was started 30 seconds after injection of 48/80 and interrupted only at end of hour and every 30 minutes thereafter. Electrocardiograms were taken 2 minutes before injection and at 1, 5, 10 and 25 minutes afterward. *Histamine.* Ten animals received a lethal dose(3) of histamine phosphate (1 mg/kg) and were given immediate artificial respiration as in group 2 above. In 5 animals electrocardiograms were taken before and up to 10 minutes after histamine.

TABLE I. Effect of Artificial Respiration on Blood Pressure Changes Induced by a Lethal Dose of Compound 48/80* in Anesthetized Rabbits.

	Before art. resp.			After art. resp.	
Min. after inj.	0	4†	6†	8	30
Mean art. press., mm Hg	118‡	148	70	130	108

* 2.25 mg/kg i.v. (1½ times LD_{50}).

† Avg time of maximum hypertension and hypotension.

‡ Each value represents mean of 22-26 animals.

Results. Compound 48/80, Group 1. In all animals after 10-60 seconds, respiration gradually declined in rate and depth, occasionally after short interval of respiratory stimulation. In 19 animals, respiratory arrest occurred in average time of 5 minutes after injection, whereas in 7 animals respiration was only severely depressed. A rise in blood pressure started shortly after onset of diminishing respiration and attained an average maximum increase of 30 mm Hg in approximately 4 minutes (Table I). The rise was followed by precipitous fall. Upon starting artificial respiration, pressure reverted to control or above control values. Subsequently when artificial respiration was periodically halted, pressure again fell, recovering only upon resumption of artificial respiration. Two animals in this group receiving artificial respiration alone, reverted within an hour to spontaneous breathing adequate to sustain blood pressure within control limits. In only one experiment in which analeptic drugs were administered was there a return of spontaneous respiration; 2 injections of pentylenetetrazol had been given 20 min. apart after first hour. Actual effectiveness of pentylenetetrazol was questionable, however, in view of some results obtained using artificial respiration alone in this group and in Group 2. Other animals, whether receiving analeptics or not, usually died after approximately one hour following brief cessation of artificial respiration.

Compound 48/80, Group 2. When artificial respiration was started almost immediately, the initial pattern of blood pressure change seen in previous group no longer occurred (Table II). In 1½ to 3 hours, 5 animals resumed spontaneous breathing sufficient to maintain blood pressure above 90 mm Hg.

These animals had not been subjected to frequent periods of anoxia as in Group 1, and were maintained for longer periods and in some instances to the point where effects of drug had begun to diminish. Electrocardiographic records revealed no significant changes in cardiac rate or rhythm during first 25 minutes after injection.

Histamine. In all animals, blood pressure began to fall precipitously a few seconds after injection, reaching zero within 3-5 minutes. Even though artificial respiration was started 30 seconds after injection of histamine and continued for 10-15 minutes, blood pressure remained at zero, no spontaneous respiration was observed at end of experimental period, and no animals survived. Electrocardiograms recorded from 5 animals, one minute after injection revealed a variety of striking changes such as marked bradycardia, prolonged P-R interval, and varying degrees of A-V dissociation with QRS complexes that were abnormal with respect to amplitude and width. One animal exhibited brief intermittent episodes of cardiac arrest. Numerous abnormalities either persisted at 5 minutes or occasionally changed from marked bradycardia and partial A-V block to ventricular tachycardia. The 10 minute records usually revealed very slow electrical activity consisting mainly of P waves and independent widened QRS complexes of greatly reduced amplitude.

Discussion. Histamine and compound 48/80 produce death in the rabbit by different mechanisms. Lethal doses of 48/80 produced a striking depressant effect on respiration, the nature of which has not been determined. All animals could be kept alive for protracted periods following doses of 48/80 several times the LD_{50} as long as artificial respiration was administered. Blood pressure changes appeared to be directly dependent on respiratory effects since artificial respiration reversed or eliminated these changes. Dews *et al.*(2) reported a rise in blood pressure of the rabbit following intravenous administration of 2 mg/kg of 48/80. It is possible that the rise was due to asphyxia.

Absence of any serious effect of large quantities of 48/80 on the cardiovascular system in artificially respired animal was further indicated by a normal ECG up to 25 minutes after injection. Failure of certain analeptic drugs to improve respiration may have been due to inadequate dosage or to the possibility that analeptics do not act upon sites affected by 48/80.

TABLE II. Mean Arterial Pressure (mm Hg) of Anesthetized Rabbits Artificially Respired Immediately after Lethal Doses of Compound 48/80 and Histamine.

	Min. after inj.			
	0	5	10	30
I Compound 48/80 (4.5 mg/kg i.v.)*	118	111	111	106
II Histamine phosphate (1.0 mg/kg i.v.)	99	0	0	--

* Dose equals 3 times LD_{50}.

Each value for 48/80 group represents mean of 8-9 animals; for histamine, mean of 10 animals.

Following a lethal dose of histamine, however, blood pressure fell rapidly to zero and death ensued despite immediate administration of artificial respiration. Death may have been caused by known constrictor action of histamine on pulmonary arterial system of the rabbit with subsequent cardiac failure(4). The marked ECG abnormalities could have been due to such an effect coupled with adverse direct action of histamine on the heart. This series of events, possibly in conjunction with peripheral vascular changes, may account for circulatory collapse and absence of any beneficial effect from artificial respiration.

Respiration in the rabbit is affected during histamine poisoning(5). The influence of histamine on respiration was difficult to assess but in view of the very rapid onset of blood pressure and cardiac changes and failure of artificial respiration as a resuscitative measure, the primary effect appears to be cardiovascular. Respiratory defects could occur concomitantly with, or as a result of, initial deleterious action on the cardiovascular system. In any case they are not the primary reversible consequence which seems to be the case with 48/80.

Schachter(6) reported that intravenous injection of smaller doses of 48/80 in rabbits caused no significant increase in plasma histamine but that the compound did release histamine from the perfused isolated ears of this animal. In our experiments doses several times the lethal dose elicited no responses which suggested release of significant amounts of histamine.

Summary. 1) In anesthetized rabbits, lethal doses of compound 48/80 produced marked depression of respiration with ensuing blood pressure changes that could be altered by administering artificial respiration. Blood pressure and electrocardiographic records in animals artificially respired soon after receiving doses several times the lethal dose revealed no evidence of adverse effects on the vascular system or on cardiac rate and rhythm. 2) Under similar conditions, however, a lethal dose of histamine produced a prompt and profound fall in blood pressure, marked changes in cardiac rate and rhythm and death despite artificial respiration. The effect appears to be primarily on the cardiovascular system. Respiratory effects are probably secondary to this main action. 3) The lethal action of 48/80 in rabbits is apparently not mediated by release of endogenous histamine since large doses do not elicit histamine-like responses.

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Co^{60} Vit. B$_{12}$ Binding Capacity of Normal Human Cerebrospinal Fluid.*†

(24715)

LEO M. MEYER, ROBERT W. BERTCHER AND CLAIRE MULZAC
South Nassau Communities Hospital, Oceanside, N. Y.

As extension of continuing investigations in this laboratory on Co^{60} Vit. B$_{12}$ combining capacity of serum and salive(1,2,3), the following study was made on ability of normal human cerebrospinal fluid to bind radiovitamin. Utilizing microbiologic assay methods, Sobotka, Christoff and Baker recently reported Vit. B$_{12}$ content of cerebrospinal fluid from normal subjects to range from 0-30 $\mu\mu$g/ml(4). These authors found elevation of Vit. B$_{12}$ in the cerebrospinal fluid and serum of about one-third of patients with multiple sclerosis.

Material and methods. Cerebrospinal fluid was obtained from 22 normal subjects with no apparent neurologic disorder, who were subjected to spinal puncture for injecting an anesthetic agent. The Co^{60} Vit. B$_{12}$ had a specific activity of 0.823 mc/mg. To successive 1 ml aliquots of cerebrospinal fluid in Visking bags were added 1, 2, 5, 5, 10, 25, 50 and 100 mμg of radiovitamin. After incubation at room temperature for 2 hours, the mixture was subjected to exhaustive dialysis in cold, running tap water for 48 hours. All specimens were set up in duplicate, one to serve as a standard. Standards and dialyzed samples were placed each in 4 ml vials, filled with concentrated sulfuric acid to dissolve the casing, and radioactivity measured in a well-type scintillation counter. "Bound" Vit. B$_{12}$ was calculated as activity retained in the bag after dialysis. Similar studies were made in 5 instances in which 1 ml of normal saline was substituted for cerebrospinal fluid, Co^{60} Vit. B$_{12}$ added, and dialysis performed as de-

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* This study was supported in part by grant from Nat. Cancer Inst., N.I.H., U.S.P.H.S.

† Co^{60} vit. B$_{12}$ was provided by Merck and Co., Rahway, N. J.

TABLE I. Co^{60} Vit. B_{12} Binding Capacity of Normal Human Cerebrospinal Fluid.

mμg Co^{60} B_{12} added/ml spinal fluid	1	2.5	5	10	25	50	100
Mean mμg Co^{60} B_{12} bound	.47	.57	.71	1.0	1.6	2.4	4.2

scribed above. Protein content of 10 samples of cerebrospinal fluid was determined by the method of Looney and Walsh(5).

Results. Table I shows mean values for Co^{60} Vit. B_{12} combining capacity of cerebrospinal fluid from 22 human subjects with no clinical evidence of neurologic disorder. Mean CSF protein value was 34.8 mg %. Table II summarizes mean results of dialysis in 5 cases in which saline was substituted for cerebrospinal fluid. Fig. 1 depicts mean spinal fluid binding capacity minus "background" of mean saline values described in Table II. For comparative purposes mean Co^{60} Vit. B_{12} binding capacity of normal human serum is shown in Fig. 1.

TABLE II. Co^{60} Vit. B_{12} in Saline Retained after Dialysis.

mμg Co^{60} B_{12} added/ml saline	1	2.5	5	10	25	50	100
Mean mμg Co^{60} B_{12} retained	.07	.09	.12	.15	.25	.5	1.0

Discussion. Previous studies suggest a 2-phase Co^{60} Vit. B_{12} binding capacity of normal human serum, with an increase of first component in chronic myelocytic leukemia, and some patients with myeloid metaplasia and polycythemia.(1,2). This phenomenon appears to be lacking in normal cerebrospinal fluid (Fig. 1). Furthermore, slope of the line describing spinal fluid binding capacity does not parallel either of the serum components. Serum binding capacity at the 1 mμg level is 2.5 times that of the spinal fluid and more than 3.5 times higher at the 50 mμg point.

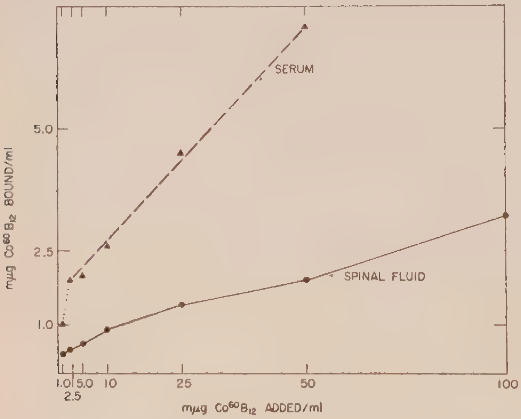

FIG. 1. Comparison of Co^{60} vit. B_{12} combining capacity of normal human serum and cerebrospinal fluid (saline values deducted).

Pitney found serum Vit. B_{12} bound to alpha globulin *in vivo* and to beta globulin when added *in vitro*(6). Pert and Kutt have reently investigated the distribution of protein fractions in cerebrospinal fluid using starch gel electrophoresis(7). Electrophoretic patterns of normal serum and concentrated spinal fluid show alpha and beta globulin peaks that are essentially the same. Consequently small amounts of protein in spinal fluid are probably not responsible for B_{12} binding in the spinal fluid unless they are in some fashion altered and have a strikingly increased number of binding sites or are potentiated by some other substance or substances present. However many other materials have the capacity to bind Vit. B_{12} and their presence or absence in spinal fluid have not been demonstrated(8).

Conclusions. 1. Normal human cerebrospinal fluid has the capacity to bind Co^{60} Vit. B_{12}. 2. This capacity to bind Vit. B_{12} increases as concentration of radiovitamin is increased. 3. Relationship of Co^{60} B_{12} binding capacity to protein content is higher for normal cerebrospinal fluid than for normal human serum.

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MSH-like Substance from Human Placenta.* (24716)

Harold H. Varon† (Introduced by Robert W. Wissler)
Dept. of Pathology, University of Chicago, Chicago, Ill.

A melanocyte stimulating hormone (MSH) from pituitary which is of functional importance in lower animals has been known for several years(1-4). Interest in this hormone was renewed in 1954 after Lerner and coworkers showed that MSH had a role in human physiology(5). This group also reported that blood and urine levels of this homone were elevated during pregnancy(6). This paper describes isolation of MSH-like substance from human placenta.

Isolation procedure is a modification of that employed by Lerner *et al.* for preparation of pituitary MSH(5), which was an adaptation of E. B. Astwood's method of extracting corticotropins from pituitary(7,8). Application of their technics to placenta will be described but details of directions will not be repeated. Full term placentas, average weight 550 g, were collected from Chicago Lying-In Hospital soon after delivery, and then extracted individually. Umbilical cord and membranes were cut and discarded. Remaining placenta together with some maternal blood was cut into squares 5 x 5 cm and placed in 1000 ml of glacial acetic acid and kept at room temperature. The entire procedure was completed within 1 hour after delivery. After 3 days, the solid material was processed with Waring blender and additional 500 ml of glacial acetic acid was added. Total mixture was then heated to 70° and cooled, and solid material discarded. To the supernatant, acetone and 5 M aqueous sodium chloride were added as described by others(5). The resulting precipitate was discarded. An equal volume of ether was added to supernatant and the precipitate was washed thoroughly with acetone, transferred to desiccator, and dried over NaOH. A tan powder was formed, 21 g in average weight/placenta. This was then treated with 20 g of oxycellulose and eluted with 0.1 N hydrochloric acid. The solution was neutralized with NaOH, subjected to brief dialysis, and lyophilized. An average of 350 mg of this material was collected from each placenta. To decrease ACTH potency, this powder was dissolved in H_2O, adjusted to pH 12.8 with NaOH, and heated 5 to 10 minutes at 90-95°(9). The solution was cooled, neutralized, and lyophilized. The final product averaged 120 mg/placenta. The assay employed was similar to *in vitro* frog skin method(10). However, the skin of *Anolis carolinensis* (chameleon) was substituted because of certain advantages(11). Selected lots of hog pituitary MSH from research labs. of Armour and Co., were used as standards. *Properties.* The material extracted was a tan powder readily soluble in water. The activity decreased greatly within a matter of weeks when stored at room temperature. As a result the material was assayed soon after preparation. Loss of activity was slowed down by refrigeration. The substance caused darkening of *Anolis carolinensis* skin similar to that caused by known hog pituitary MSH. When the skin is placed in a solution containing the material, there is continual darkening for 20 minutes after which some paling takes place. The darkening is reversed if the skin is placed in Ringer's solution.

Results of assay. The results listed in Table 1 were obtained from assay of MSH preparations from 10 placentas. Average concentration was about 3.05 x 10^2 units/mg. This activity is much less than the reported value for pure MSH from hog pituitary of 5 x 10^7 units/mg(5). Our material had a potency 1/200-1/300 of MSH preparations from hog pituitary which we tested.

Discussion. Because the placenta is an organ with large blood volume (estimated 10% plasma)(12), this contribution is a matter of concern. The highest reported value for MSH in serum during pregnancy is 7.9 U/ml (or

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* This work was partially supported by grant from Am. Cancer Soc.

† Present address: Naval Med. Research Inst., Bethesda, Md.

TABLE I. Assay of MSH Preparations from 10 Placentas from 10 Patients.

U*/mg	U*/mg
3.60×10^2	2.65×10^2
2.75 "	4.80 "
1.30 "	2.70 "
4.00 "	3.20 "
3.10 "	2.40 "

* Unit of activity is defined as degree of darkening, measured with photoelectric reflection meter, produced by .04 μg of lyophilized water extract of beef pituitary powder on isolated frog skin(10).

about 7.9 U/g)(6). This makes a hypothetical placenta: whole blood gradient of more than 30,000:1. This material seems present in concentrations greater than can be accounted for by its being derived from placental blood.

This study does not differentiate between placenta as site of storage or site of production of an MSH-like substance. Experiments attempting to answer this question are projected.

It is known that melanocyte stimulating activity is intrinsic to corticotropins. In this study it was noted that after alkalinization and heating, which destroys much of the corticotrophic activity(9), the melanocyte stimulating activity was enhanced 20 to 30 times. In addition, the darkening was qualitatively somewhat different, with a prolonged and slower darkening caused by the alkali treated material.

Conjecture regarding the biological significance of placental MSH may be summarized as follows: The rapid decline of MSH values in blood and in urine soon after delivery (6) might be due to loss of the placenta containing large quantities of this substance. Whether the formation and rapid post partum loss of much of the increased dermal and mucosal pigmentation commonly noted during pregnancy (chloasma) is also related to placental MSH requires further study.

Conclusion. An MSH-like substance was present in considerable concentration in human placenta.

I am grateful to Dr. R. W. Wissler for encouragement and support, to Dr. R. Stoughton for helping initiate this work. Research Laboratories of Armour & Co. were extremely generous in providing material. The following have kindly given me criticism and suggestions: Dr. Z. Hruban, Dr. E. J. Plotz, Dr. J. Fisher and Mr. L. Frazier.

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Prevention of Viral Lymphoid Leukemia of Mice by Thymectomy.[*][†] (24717)

J. D. LEVINTHAL, R. F. BUFFETT AND J. FURTH
Children's Cancer Research Fn., Boston, Mass.

Most animals of the high leukemia mouse strains Ak and C58 die of spontaneous leukemia, and all transmit the genetic liability for leukemia to their offspring, whether or not the parents die of leukemia. These genetically determined leukemias originate in the thymus

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[†] We acknowledge the skillful technical assistance of Mrs. Sah Sook Cho.

for the most part, and are preventable by thymectomy. Virus-like agents capable of inducing leukemia have been isolated from these high leukemia strains. An experiment was set up to determine whether thymectomy will prevent induction of leukemia by a virus originally derived from Ak leukemia tissue.

Material and methods. Virus. The passage virus, "Agent A" of Gross(1) was used. Filtrates produce about 50% leukemias in C3H/f/G recipients in less than 6 months after injection in the newborn period, and extracts produce an even higher percentage with a shorter latent period(1). Our material was derived from 2 animals (one each from passages 10 and 11) sent to us by Dr. L. Gross in November, 1957. A filtrate was prepared from the pooled leukemic tissues of these 2 mice and frozen at –70°C. Vials containing 1 cc aliquots were thawed as needed and injected into 112 C3H/f/B/G/L‡ (hereafter designated Z) baby mice less than 7 days of age (passage 12). Two leukemic mice from passage 12, one injected <40 hours, the other <8 hours of age, were sacrificed at 77 and 111 days respectively. A filtrate was prepared from each animal (passage 13) and stored in sealed ampoules at –70°C until used. *Filtrate preparation.* Lymph nodes, a piece of liver (excluding gall bladder), spleen and thymic tumor of leukemic mice were harvested aseptically, ground in a chilled mortar, diluted to a 15-20% suspension with chilled normal saline and centrifuged at 0°C for 15 minutes at 1500 g. The fatty scum occasionally found on the surface was removed with a sterile swab, and the supernatant fluid centrifuged at 6,900 g for 10 minutes at 0°C. The second supernatant fluid to which *E. coli* was added was passed through a chilled Selas 02 filter previously moistened with chilled normal saline. Cultures of material before filtration had abundant growth, while the filtrate was sterile. The filtrate was divided into 1 cc aliquots in small vials, sealed and stored at –70°C, after first chilling for half an hour in the freezing compartment of an ice box. Vials were thawed as needed during 2 months after preparation. *Treatment of mice.* A total of 187 Z mice were injected i.p. with 0.025 to 0.07 cc at less than 7 days of age with these 2 filtrates (passage 13). Of these, 99 survived 60 days. Since there was no marked difference in infectivity of these 2 filtrates the data are combined. Alternate litters were set aside as controls. At about the age of 4-6 weeks, alternate animals in each injected litter were *thymectomized* under ether-nembutal anaesthesia. Fourteen alternate uninjected controls were also thymectomized. Intercurrent infections in all animals were treated by the addition of veterinary oxytetracycline or chloramphenicol to the drinking water, and in some cases by injection of a streptomycin-penicillin mixture. Males and females were caged together. Babies born to virus-injected animals were destroyed after birth; those from uninjected controls were raised. All animals that died were autopsied and tissues of many leukemic and non-leukemic mice were examined histologically. At 7 to 9 months of age all surviving animals were sacrificed, autopsied, and many normal appearing mice studied histologically. In addition, bioassays of normal appearing thymuses and lymphoid tissues of virus injected and uninjected control animals, selected at random, were performed for presence of leukemic cells and possible persistence of virus. The bioassay (grafting lymphoid tissues on histocompatible normal young adults) is in progress.

Results. There were 25 leukemias among 50 virus-injected intact mice but only 1 in 49 virus-injected thymectomized mice, and none in uninjected controls. Fig. 1 shows total cumulative leukemia mortality in virus injected animals. All but one of the 25 leukemias found in virus-injected intact animals had a thymic tumor, most of these with gross enlargement of the spleen and involvement of lymph nodes.

No other tumors or evidence of "polyoma" virus(2) activity were found in spite of search by both gross and microscopic examination of salivary glands, thymus, kidney and other organs.

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‡ This sybmol is that recommended by G. Snell, indicating C3H mice, foster nursed, originally obtained from Bittner and carried for several generations by Gross and his subline by Levinthal.

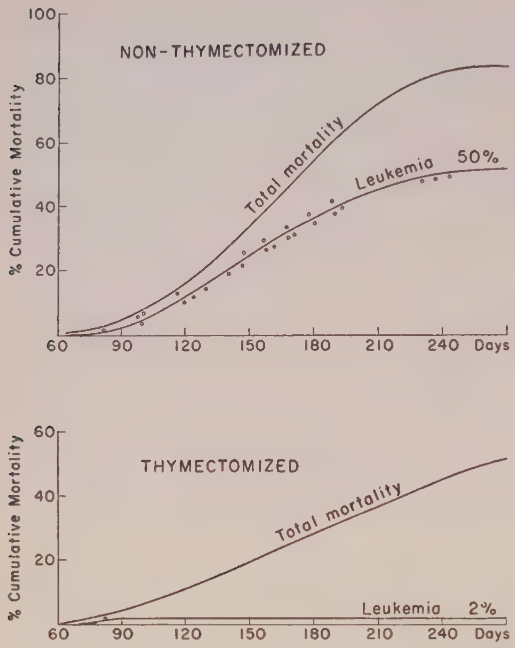

FIG. 1. Cumulative total and leukemia mortality (% of total group) in thymectomized and non-thymectomized virus infected Z mice. On leukemic line each dot represents a leukemic mouse.

Table I gives the cumulative mortality and leukemia incidence in all experimental and control groups in this study.

Passage 13 virus caused somewhat fewer leukemias than passage 12, but this can be explained mainly by intercurrent pneumonia of an interstitial non-bacterial type. In general, the difference in mortality among the infected groups was due to leukemia, the non-leukemic mortality being comparable in all 4 groups (Table I).

In both passages, there was a slightly higher incidence of leukemia in females (average 59%) than in males (54%) and a slightly shorter latency period (Table II), regardless of age at time of injection.

These findings parallel the observations on spontaneous leukemia in Ak mice(6) in which the frequency is greater in females and is markedly reduced by intercurrent pneumonia. Reduction of leukemia frequency by pneumonia can be attributed to partial thymic involution due to stress, mediated by the adrenal cortex. This has been duplicated by direct administration of cortisone.

Fig. 2 shows the data on infected non-thymectomized mice and controls in both passages indicating death from leukemia and other causes, distinguishing between animals found dead and sacrificed at termination of experiment. On basis of available information termination of such experiments at 240-270 days, as done here, is permissible for first approximation. Earlier studies have indicated that the leukemias occurring at later age are not thymic and are not influenced by thymectomy(6).

Discussion. Agents capable of inducing leukemia in genetically susceptible strains of mice include whole body irradiation, carcinogens such as methycholanthrene, and viruses

TABLE I. Cumulative Mortality from Leukemia and Other Causes.

Mice	No. in group†	Age, days													
		61-90		91-120		121-150		151-180		181-210		211-240		241-270	
		L*	T*	L	T	L	T	L	T	L	T	L	T	L	T
Passage 12															
I Virus infected non-thymectomized	77	1	1	8	10	23	28	33	39	42	49	46	56	46	57
II Non-infected	78	0	1	0	3	0	3	0	8	0	11	0	21	0	25
Passage 13															
I Virus infected															
(a) Thymectomized	49	1	2	1	7	1	9	1	14	1	17	1	23	1	25
(b) Non-thymectomized	50	1	3	6	9	11	18	19	29	22	37	24	41	25	42
II Normal, uninj.															
(a) Thymectomized	14	0	2	0	3	0	5	0	6	0	6	0	6	0	8
(b) Non-thymectomized	27	0	2	0	2	0	6	0	7	0	10	0	10	0	12

* L = leukemia mortality; T = total mortality.

† Discounting those dying younger than 60 days of age.

TABLE II. Viral Infectivity in Relation to Sex.

	Males			Females		
	No. L/No. inj.	%	Latency, days (avg)	No. L/No. inj.	%	Latency, days (avg)
Passage 12	25/43	58	168	21/34	62	148
" 13	12/26	46	169	13/24	54	148
Combined	37/69	54	168	34/58	59	148

(1.3.4.5). The virus of Gross and the non-viral agents usually mediate their effect through the thymus. Thymectomy reduces incidence of spontaneous leukemia in the high leukemia strains Ak(6), and C58(7) in which leukemia has been related to both genetics and virus. Similarly, thymectomy prevents development of thymic lymphomas following whole body irradiation(8) or administration of methycholanthrene(9). Thymic homografts in thymectomized mice of low leukemia strains reestablish the increased leukemia incidence which follows administration of methycholanthrene(9) or x-rays(10).

The present experiment indicates that the virus derived from Ak material requires the thymus to cause leukemia, within the time limit of our study.

By what mechanism thymectomy prevents leukemia is still to be established. Numerous workers noted that the thymus has a lymphocytosis stimulating factor (LSF). As early as 1909, Maximow(11) observed that the thymic epithelial anlage attracts lymphocytes, and stimulates their proliferation within the organ. This work was carried further by Jolly(12), Gregoire(13), Law(14), Kaplan (15), and Metcalf(16). Gregoire(13) noted that implants or extracts of thymic epithelial tissue (heterologous thymus depleted of thymic lymphocytes by irradiation, leaving the radio-resistant epithelial stroma) stimulated lymphopoietic activity in regional lymph nodes while implants of lymph nodes or musle fragments were ineffective. Metcalf(16) correlated the high level of circulating lymphocytes in the high leukemia strains Ak and C58 with a greater blood level of LSF during their preleukemic period than in low leukemia strains. But this does not explain why the leukemic lymphocytes behave as autonomous cells; excessive stimulation should cause lymphocytosis, *i.e.*, an increase of normal but not autonomous cells.

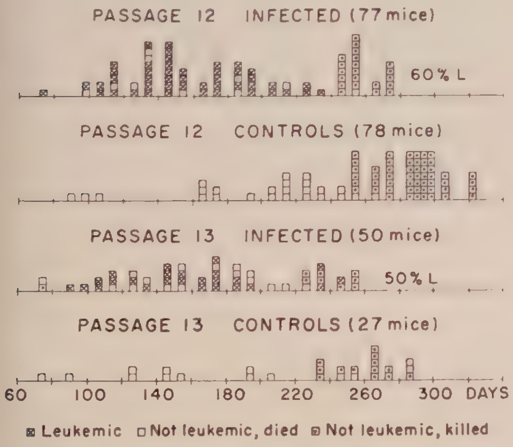

FIG. 2. Histogram of infected (not thymectomized) mice and controls in passages 12 and 13 indicating death from leukemia and other causes and distinguishing between animals found dead and sacrificed at termination of experiment.

The question is, therefore, what brings about autonomy? The apparent autonomy of viral leukemia is indicated by its transplantability. Is autonomy due to mutation of thymic lymphocytes under the influence of virus, x-rays or carcinogens? Is this influence localized in the thymus because of a genetic affinity of this organ to virus, or because of LSF, which is produced by the thymic epithelial reticulum and is in proximity with thymic lymphocytes? Is a specific growth factor required for leukemic transformation of lymphocytes? The latter hypothesis is suggested by the work of Law(14) indicating that the lymphocytes of some lymphomas arising in thymic grafts originate in the host.

If direct cancerization of thymic lymphocytes by virus is postulated (without the aid of LSF), one must assume that thymic lymphocytes are different from other lymphocytes, and that thymectomy achieves physical removal of lymphocytes most susceptible to malignant transformation. If a 2-stage process is postulated (virus and LSF), it may be possible to induce lymphoma by virus in lymph nodes by providing adequate concentration of LSF. A third possibility is that the thymic epithelial reticulum is an especially good culture medium for the virus, and that a high concentration of virus is necessary for leukemia induction.

Data presented by Gross suggest the assumption that the virus causing lymphoma in Ak mice is different from that causing multiple tumors. The observations of the present studies in conjunction with those of Buffett *et al.*(17), carried out simultaneously in this laboratory, strongly support this assumption. A critical experiment in which these 2 different materials with viral activity were injected in the same type of host, now in progress, supports this view.

Earlier experiments(6) have shown that transplantation of leukemic cells is not influenced by absence of thymus. Therefore, prevention of leukemias in thymectomized animals demonstrates that leukemic cells did not pass through the filters.

Summary and conclusions. 1. Ninety-nine Z mice were injected with Gross "Agent A" (Selas filtrates). Leukemia was induced in 1 of 49 animals thymectomized at 4-6 weeks of age, and in 25 of 50 alternate non-thymectomized mice. No leukemias occurred in 41 uninjected controls. The experiments were terminated when the animals were 7-9 months of age. 2. Incidence of leukemia induced by virus was greater and the latent period shorter in females than in males. 3. Spontaneous Ak leukemia, from which this agent is derived, behaves similarly to viral leukemia with respect to relaton of sex to incidence and latency period. However, spontaneous leukemia occurs at a later age than this viral leukemia. 4. The thymus is the fulcrum of both lymphoma induction by this virus and of its spontaneous development in Ak mice; its presence is essential for development of this type of leukemia.§

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§ Since this paper was submitted, we have learned of similar results in Ak mice obtained by Dr. J. F. A. P. Miller of Chester Beatty Research Institute, submitted to *Nature*.

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Acute Effect of 6 Methyl Prednisolone (Medrol®) on Impaired Water Excretion of Adrenal and Pituitary Insufficiency.* (24718)

CHARLES R. KLEEMAN, JERRY KOPLOWITZ AND MORTON H. MAXWELL
Wadsworth Hospital, Veterans Admin. Center, and Dept. of Medicine,
University of California, Los Angeles

An impaired diuretic response to water is a characteristic abnormality of patient or animal with adrenal or pituitary insufficiency (1-6). This defect can only be corrected by administration of hydrocortisone-like steroids (3-6), probably by direct effect on kidney rather than by extra-renal mechanisms(4,7,9-12). Hydrocortisone increases glomerular filtration, renal blood flow and enhances tubular reabsorption of sodium in the adrenal and pituitary insufficient subject(7-12). However, our previous studies indicate that marked improvement in water diuresis caused by compound F cannot be explained solely by its effects on renal hemodynamics or solute excretion(7). It seemed therefore of interest to note the effect of Medrol® (6 methyl prednisolone) on impaired diuresis of adrenal and pituitary insufficiency. This potent adrenal cortical analogue does not enhance sodium reabsorption or alter renal hemodynamics in normal subjects(13).

Materials and methods. Six experiments were performed in 6 male patients: 4 with anterior pituitary insufficiency (secondary adrenal insufficiency) and 2 with primary adrenal insufficiency. Hydrocortisone replacement therapy was withdrawn from all patients for at least 1 week prior to experiments. Subjects with primary adrenal insufficiency were maintained on 100 μg of 9-alpha fluoro-hydrocortisone daily. Experiments were performed in the morning post-prandially, with subjects recumbent. An initial urine specimen was obtained for osmolality, after which an oral water load of 1000 cc was given; this positive water balance was maintained for duration of experiment by infusion of 2.5% glucose in water at a rate equal to urine flow. Collection periods were 20 to 30 minutes, and urine samples collected by spontaneous voiding or by indwelling catheters. When urine volume was below 2 cc/minute, air "wash outs" were also utilized. Samples of venous blood were obtained at midpoint of each period through an indwelling needle. Control collections were begun about 45 minutes after priming dose of inulin and para amino-hippurate and constant sustaining infusion had been started. After at least 3 control periods of maximally sustained urinary flow had been obtained (approximately 2½ hours after initiating the water load), 5-20 mg of Medrol® were administered into infusion tubing for 30 minutes. Urinary collections were continued for additional 3 to 5 hours. Experiments ranged from 5½ to 8 hours in duration. Analysis of serum and urine for sodium, potassium, chloride, inulin, PAH, creatinine, and freezing point depression (osmolality: milliosmols/kg of H_2O) were made utilizing methods previously reported(14,15). Characteristics of water diuresis were evaluated in each case by maximal urinary flow, minimal urinary osmolality and magnitude of free water clearance C_{H_2O}.† Since the experiments were too rigorous to permit *control* water loading studies *without drug administration* on each subject, urine volumes were measured in 3 other patients (2 pituitary and 1 adrenal insufficiency) maintained in sustained positive water balance for 5 hours; in these 3 control subjects, maximal urinary flows were attained in the first 2½ hours.

Results. The significant data from each study are presented in Table I and a representative experiment is outlined in Table II.

A. *Response to Water Loading*: Although

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† C_{H_2O} is equivalent to the volume of urine/minute virtually freed of solute during diluting process. It represents the difference between total urinary flow, V, and volume of fluid necessary to excrete all urinary solutes in an isosmotic solution, or osmolal clearance, $Cosm: V - Cosm = C_{H_2O}$.

TABLE I. Effect of Medrol® on Water Diuresis in Adrenal and Pituitary Insufficiency.

Subject	Period	Urine vol, ml/min.	Urine conc., mOsm/kg	UV sol,* μOsm/min.	C_{H_2O},† ml/min.	GFR,‡ ml/min.	RPF,§ ml/min.	$U_{Na}V$, μeq/min.	Serum osmolal., mOsm/kg
Adrenal insuff.	Control	2.8	298	834	− .4	68	275	144	264
	Medrol	10.0	100	1007	+6.4	70	277	257	274
Idem	Control	4.0	275	1100	− .1	60	240	560	267
	Medrol	10.3	62	638	+7.8	87		540	258
Ant. pit. insuff.	Control	1.2	432	518	− .7	80		128	285
	Medrol	9.8	109	1068	+5.5	100		281	257
Idem	Control	1.2	410	508	− .6	105	419	81	283
	Medrol	6.6	113	746	+4.0	105	414	112	277
"	Control	.9	787	716	−1.5	95	350	162	294
	Medrol	10.0	98	984	+6.7	94	419	221	290
"	Control	2.1	410	853	−1.3	84	341	250	254
	Medrol	7.0	120	840	+3.6	86	327	355	250

* Rate of solute excretion. † Free water clearance. ‡ Glomerular filtration rate. § Renal plasma flow.

maximal urinary flow was attained after time interval comparable to normal subjects, the magnitude of peak diuresis was distinctly subnormal (mean urinary flow 2 cc/min., range 0.9-2.8 cc/min.; mean urinary osmolality 435 mOsm/kg, range 275-787 mOsm/kg) and mean C_{H_2O} –0.9 cc/min., (range –0.4 to 1.5 cc/min.). Glomerular filtration rate (inulin clearance) and renal plasma flow (PAH clearance) were below normal in all subjects, when referred to surface area of 1.73m².

B. *Response to Medrol®*. A noticeable increase in urinary flow and fall in urinary osmolality occurred within 2½ hours after administration of the steroid, while peak response was observed in 4 to 5 hours (Table II). The difference from control periods was striking. Urinary flow increased to a mean of 9 cc/min. (range 6.6-10.3 cc/min.), C_{H_2O} to a mean of +4.2 cc/min. while urinary osmolality fell to 100 mOsm/kg (range 62-120 mOsm/kg). Sodium and total solute excretion increased moderately in 5 of the 6 subjects. Although inulin clearance increased significantly in 2 subjects, there was no correlation between magnitude of improvement of water diuresis and increment in glomerular filtration rate.

Discussion. These studies clearly indicate that acute administration of 5 to 20 mg of Medrol® (6 methyl prednisolone) can markedly improve all parameters of the impaired water diuresis associated with adrenal and pituitary insufficiency. This effect is comparable in all respects to that observed follow-

TABLE II. Effect of Medrol® on Impaired Water Diuresis of Patient.

Time	Urine vol, ml/min.	Urine conc., mOsm/kg	UV sol,* μOsm/min.	C_{H_2O},† ml/min.	GFR, ml/min.	RPF, ml/min.	$U_{Na}V$, μeq/min.	Serum osmolal., mOsm/kg
60	.8	922	738	−1.6	107		154	307
90	.8	867	711	−1.6	87	375	143	293
120	.9	789	686	−1.6	95	350	139	
150	.9	787	716	−1.5	103	350	162	294
‡180	.8	818	638	−1.4	91		147	
210	.7	820	607	−1.3	96		139	295
240	.8	820	672	−1.3	196		145	
270	1.0	790	797	−1.8	120	403	167	285
300	1.4	686	960	−1.9	115	455	221	
330	2.6	380	969	− .8	99	420	176	289
360	5.4	176	956	+2.1	93	421	195	
390	8.5	116	988	+5.1	92	397	222	290
420	10.0	98	984	+6.7	94	419	221	

* Rate of solute excretion. † Free water clearance. ‡ 20 mg of Medrol® given intrav. at start of this period.

ing administration of 100-200 mg of hydrocortisone to similar patients[‡](7). It is readily apparent that this response was not dependent on increased glomerular filtration rate or renal plasma flow (Table I, II), although it seems reasonable that any improvement in renal hemodynamics would enhance the diuretic response(16).

Medrol® caused a net decrease in tubular reabsorption of sodium and a slight increase in solute excretion in these subjects (Table I). These alterations could not have appreciably enhanced urinary dilution or flow.

If, as available data suggests, compound F like steroids does not alter metabolism of antidiuretic hormone(4,7,9-13), the present studies lend further support to the concept that these steroids improve water diuresis by inhibiting the back diffusion of water in diluting segments of the nephron; a permissive action.

Summary. Acute administration of 6 methyl prednisolone (Medrol®) can markedly improve impaired water diuresis of adrenal and pituitary insufficiency. This effect is not dependent on altered renal hemodynamics or sodium reabsorption, and it suggests a specific action on the diluting segment of the nephron.

[‡] In adrenal and pituitary insufficient patients as little as 3 mg of Medrol®, administered intravenously 1 hour prior to oral injection of 1500 cc of water, can completely correct impaired response to this load(14).

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Glucocorticoids and Maintenance of Blood Pressure and Plasma Volume of Adrenalectomized Dogs Subjected to Stress.* (24719)

W. W. SWINGLE, J. P. DAVANZO,[†] H. C. CROSSFIELD,[†] D. GLENISTER, M. OSBORN, R. ROWEN AND G. WAGLE

Biological Laboratory, Princeton University, Princeton, N. J.

Mild stress is rapidly fatal to adrenalectomized (adx) dogs adequately maintained on desoxycorticosterone or high salt therapy, whereas animals with intact cortices are extremely hardy and withstand severe stress. Moreover, the adx dog primed with cortical extract(1), with cortisone or hydrocortisone, becomes refractory to traumatic or other deleterious stimuli(2). Intravenous infusions of those steroids with an oxygen function at Carbon 11 and 17, or their more potent analogs,

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[†] N. J. Heart Assn.

e.g., 2-methyl-9α fluorohydrocortisone (2-methyl FF) and 1-dehydrohydrocortisone (prednisolone, 1-dehydro F), restores normal activity and vigor to the animal in collapse from either adrenal insufficiency or as a result of some type of stressful situation. The results recorded here on adx dogs subjected to 2 types of stress, are suggestive and possibly afford a clue to the nature of certain factors which greatly enhance the sensitivity of this type of animal to harmful stimuli.

Methods. Vigorous adx dogs were used; they had been studied through numerous cycles of insufficiency and recovery. Maintenance was effected by daily i.m. injections of 0.5-0.7 mg of DCA, plus salt supplement of 1 g NaCl/day. Steroid and salt were discontinued and food withheld 24 hours previous to starting an experiment. After application of stress, the animals' bladders were drained by catheter and the dogs placed in metabolism cages *without food or water for duration of experiment.* Methods for determination of blood pressure, plasma volume and electrolytes and for solubilizing the steroids used, have been described(3,4). The animals subjected to intestinal manipulation were anesthetized with ether and a 3 inch slit made in ventral abdominal wall through which small loops of intestine were successively exteriorized and gently pinched and stretched until the length of small gut had been manipulated 20 minutes. Animals possessing intact adrenals suffer no gross ill effects when given identical treatment and upon recovery from the anesthetic, exhibit normal activity and vigor, and when fed the following day display no impairment of appetite or ability to digest their usual food ration. On the other hand, the adx dog develops fatal shock within 4-15 hours after operation unless infused i.v. with potent glucocorticoid. A convulsant drug, metrazol, was chosen as convenient stressor agent to induce circulatory failure without tissue injury or external fluid loss. Each of the 2 dogs received 250 mg by vein and was forcibly restrained during the convulsive phase of the reaction. Blood samples were drawn 15 minutes after giving the drug and repeated when the animal was in shock and again when recovery was complete.

Results. 1. *Intestinal manipulation. Untreated and treated dogs.* Two untreated and one treated animal died; they survived 7-11 hours after trauma. Arterial pressure slowly declined, terminating in circulatory collapse. Shock-like symptoms appeared 4-5 hours after operation and during this interval average mean blood pressure fell from 120 to about 60 mm Hg. Severe hemoconcentration was present and plasma volume decreased 36 to 56%. Removal of blood for sampling (37 cc) when blood pressure registered 58-65 mm Hg precipitated grave symptoms followed by death within a few hours. There was no discernible loss of fluid into intestine or body cavity. Data obtained from these animals are not recorded in the Tables. The 2 untreated dogs succumbed before treatment was initiated and the one animal that received glucocorticoid (8 mg), was moribund when the steroid was injected.

Data obtained from 4 adx dogs treated with 2-methyl - 9α-fluorohydrocortisone (2-methyl FF), are given in Table I. Two dogs of this series (Nos. 1, 2), received injections when marked symptoms were evident and while blood pressure ranged between 70-73 mm Hg. The other 2 animals (Nos. 3, 4), were bled for samples when arterial pressures were 83 and 86 mm Hg respectively. Neither of the latter showed other than mild symptoms and their plasma volume reductions were minor—approximately 6-7 cc/kg. There is grave danger of losing the animal if blood samples (37 cc) are taken when arterial pressure has fallen to 55-60 mm Hg, since the intestinal-manipulated dog is quite sensitive to small blood losses when plasma volume is low (Table I).

The rejuvenating effect of the 11-oxy steroid on activity and vigor of the 4 dogs was obvious; arterial pressure slowly rose to control or near control values, blood became dilute and plasma volume increased an average of 51% above the low level recorded at time of injection. The jugular, saphenous and other superficial veins, which before steroid treatment were collapsed and contained little free-flowing blood, became turgid and plainly visible 8-10 hours after administering 2-methyl

TABLE I. Plasma Volume and Hemoconcentration Changes of 4 Adrenalectomized Dogs Shocked by Intestinal Manipulation and Treated with Glucocorticoids.*
(Dogs 1-4)

Hr after operation	Condition of animal	Body wt, kg	BP, mm Hg	Blood urea N, mg %	Hb, g %	Hmct, %	RBC 10^6, mm^3	Blood sugar, mg %	Plasma vol		Total dose steroid, mg
									cc/kg	% change	
Initial	Normal	18.97	115	21.7	11.1	31.1	5.05	81.5	52.7		
7	Shock		70	16.3	12.5	36.0	5.73	73.0	39.4	−25.2	16
31	Normal	18.63	94	13.2	9.1	27.8	4.00	104.5	61.6	+56.3	
Initial	Normal	25.80	108	16.5	11.0	31.1	4.67	82.5	47.1		
10	Shock	25.90	73	15.7	13.9	40.7	5.68	84.5	32.0	−31.0	15
34	Normal	25.30	89	14.8	12.1	31.0	4.32	110.5	48.5	+51.5	
Initial	Normal	22.61	125	13.1	12.4	34.0	5.69	79.0	44.0		
12	Mild shock	22.15	83	13.5	13.8	35.5	5.94	66.0	37.4	−15.0	13
36	Normal	21.03	108	12.5	8.9	26.1	4.36	102.0	51.4	+37.4	
Initial	Normal	15.65	108	23.4	13.6	38.1	5.22	85.0	47.2		
7	Mild shock	15.76	86	25.7	15.4	40.9	5.36	77.5	39.7	−16.0	12
31	Normal	14.74	98	22.4	10.5	30.0	4.52	98.0	63.5	+59.9	

− = Decrease in plasma vol from normal to shock level before steroid. + = Increase in plasma vol from shock level to normal after steroid.

* 2-methyl-9α-fluorohydrocortisone.

FF. The usual diuresis occurred with Na and Cl retention and K excretion. There was evidence of Na diuresis in interval between application of trauma and steroid therapy, although this was not reflected by lowering of plasma electrolytes. Data concerning the latter are not included in the Tables since they revealed no significant alteration. Thus these ions behave differently in this respect from the striking changes they undergo in the adx dog suffering from insufficiency where Na and Cl depletion is severe but requires 7-10 days to accomplish after withdrawal of DCA. Arterial pressure in shocked dogs seemed dependent upon plasma volume; when the latter was low, the pressure was also low but rose steadily with increase in volume of fluid in circulation and rehydration of the extracellular compartment by fluid shifted out of cells (Table I).

2. *Comparison of efficacy of aldosterone and 2-methyl-9α-fluorohydrocortisone as therapeutic agents for reviving a fasted dog from circulatory failure induced by intestinal manipulation.* Previous work(4), showed aldosterone relatively inefficient as a restorative agent for *fasted* dogs exhibiting severe adrenal insufficiency, hence it was of interest to test its ability to restore the failing circulation resulting from trauma. The pertinent data from the aldosterone-treated animal are shown in Tables IIA and B. Mild symptoms were present 6 hours after trauma; blood pressure had fallen 34 mm Hg and plasma volume had decreased 37.7%. Eight mg of solubilized aldosterone were injected i.v. in 3 doses as

TABLE IIA. Ineffectiveness of Aldosterone for Reviving Fasted Adrenalectomized Dog #5 from Mild Shock Induced by Intestinal Manipulation.

Hr after operation	Condition of animal	Body wt, kg	BP, mm Hg	Blood urea N, mg %	Hb, g %	Hmct, %	RBC 10^6, mm^3	Blood sugar, mg %	Plasma vol	
									cc/kg	% change
Initial	Normal	22.91	116	19.5	9.71	31.1	4.82	85.8	51.6	
6	Mild symptoms	22.87	82	15.2	11.82	33.6	5.02	86.5	32.1	−37.7
Aldosterone—8 mg, 6 to 15th hr										
15	Shock	22.27	66	24.4	13.47	36.6	6.09	72.0	32.6	−36.8
2-Methyl FF—20 mg, 15th to 35th hr										
35	Recovery	21.25	96	17.4	10.36	29.8	4.47	115.5	54.1	+66.9

− = Decrease in plasma vol from normal to shock level before steroid.
+ = Increase in plasma vol from shock level to normal after steroid.

TABLE IIB. Plasma and Urine Electrolytes of Fasted Adrenalectomized Dog #5 Subjected to Intestinal Manipulation and Treated with (A) Aldosterone and (B) Glucocorticoid (2-Methyl-9α-fluorohydrocortisone).

Hr after trauma	Condition of animal	Plasma electrolytes, meq/l			Urine vol, cc	Urine electrolytes, meq		
		Na	Cl	K		Na	Cl	K
0	Normal	147.0	118.0	4.16				
6	Mild symptoms	152.5	117.3	3.57	86	14.49	6.12	5.54
A. Aldosterone—8 mg, 6th to 15th hr								
15	Shock	150.0	116.2	3.57	80	1.18	.90	3.42
B. 2-Methyl FF—20 mg, 15th to 35th hr								
35	Recovery	146.5	117.9	3.57	396	24.15	15.24	19.30

follows: 4, 2 and 2 mg over the next 9 hours. No improvement in animal's condition followed aldosterone treatment; instead the symptoms grew progressively worse and were accompanied by a sharp fall in blood pressure and increased hemoconcentration. Plasma volume remained essentially unchanged at the low level of approximately 32 cc kg. Plasma Na showed some increase presumably due to hemoconcentration. Also an additional 80 cc of urine containing very small amounts of Na, Cl, and K, were excreted. The dog collapsed shortly after removal of blood for sampling, and 10 mg of 2-methyl FF were given i.v. in one injection followed by an additional 10 mg injected in 2 equal doses over the next 24 hours. The prognosis on this dog was so grave that he was not expected to survive. However, 20 hours after giving glucocorticoid (35 hours after trauma), he had fully recovered. Blood volume and blood pressure had risen to control values despite lack of food and water and further loss of 396 cc of fluid, as urine during recovery. Plasma Na declined slightly, probably due to hemodilution (Table IIB).

3. *Induction of circulatory failure in fasted adrenalectomized dogs by metrazol and recovery on glucocorticoid therapy.* Two adx animals were injected i.v. with 250 mg of metrazol and immediately exhibited copious salivation and severe convulsions. They were unconscious for 2-3 minutes after which recovery was complete and uneventful. Fifteen minutes later blood was drawn for testing; during this interval, arterial pressure, pH, plasma volume and bicarbonate declined sharply (Table III). Signs of circulatory failure were observed in Dog 7, eleven hours after metrazol injection; Dog 6 required 24 hours before grave symptoms of shock appeared. However, depression and lethargy persisted in these animals from the time of drug administration. Dogs possessing adrenals show no harmful after-effect of metrazol when the convulsive reaction has passed. Such is not the case when the drug is used on the adx animal maintained on mineralocorticoid, for irreversible fluid changes are initiated which later result in circulatory failure in the untreated animal. Unequivocal signs of shock became obvious

TABLE III. Plasma Volume and Hemoconcentration Changes of 2 Adrenalectomized Dogs Following Administration of 250 mg Metrazol and Recovery on Glucocorticoid Therapy (1-dehydrohydrocortisone). (Dogs 6 and 7)

Interval after metrazol inj.	Condition of animal	Body wt, kg	BP, mm Hg	Blood urea N, mg %	Hb, g %	Hmct, %	RBC 10^6, mm^3	Plasma vol		pH	$^-HCO_3$	Steroid dose, mg
								cc/kg	% change			
0	Normal	21.70	140	15.5	12.28	34.3	5.52	46.9		7.40	39.6	
15 min.	Depressed	21.70	86	37.0	14.55	41.9	6.17	31.4	− 32.0	7.00	11.2	
24 hr	Shock	21.13	70	71.0	14.73	38.8	6.27	26.9	− 42.6	7.40	35.8	
48 "	Recovery	20.00	100	34.6	11.19	33.1	5.24	42.6	+58.2	7.35	32.8	100
0	Normal	15.42	112	14.7	13.33	38.1	5.25	52.3		7.38	44.5	
15 min.	Depressed	"	80	34.0	14.73	48.0	6.30	31.8	− 39.1	7.18	16.1	
11 hr	Shock	"	68	36.4	13.89	42.7	5.62	33.3	− 38.2	7.35	42.5	
35 "	Recovery	15.40	95	14.4	10.90	33.2	4.95	52.4	+64.4	7.31	43.4	100

11-24 hours after giving metrazol. At this time arterial pressure registered 68-70 mm Hg and plasma volume had diminished 38-42%. A further decline in blood pressure had taken place since the first determinations were made (at 15 minutes). Apparently little, if any, intracellular fluid had been shifted into the dehydrated extracellular compartment during the 24 hour interval between sampling. The clinical condition of the dogs deteriorated rapidly after drawing blood samples when plasma volume was low. It became necessary to administer the water soluble sodium hemisuccinate of 1-dehydrohydrocortisone (Intravenous Meticortelone, Schering). The initial large 50 mg dose chosen was arbitrary; a lesser amount would probably have served equally well. During the following 24 hours, two 25 mg doses were given i.v. Recovery was rapid; blood pressure, plasma volume, strength and vigor returned to normal and urine volume was much increased. Blood sugar increased during the convulsions and remained elevated throughout the experiments. It should be noted that the animals were restored to health by administering a relatively pure glucocorticoid, noted for its natriuretic rather than Na-retaining properties (Table III).

Discussion. The greatly increased susceptibility of adx dogs to stressor agents or procedures is due, apparently, to rapid inspissation of the extracellular compartment owing to passage of large volumes of water into cells as a consequence of any non-specific untoward stimulus. The fluid so shifted is held immobile within the cells presumably by osmotic forces which the animal is powerless to overcome in absence of glucocorticoids, despite the desperate need to rehydrate his extracellular space and maintain circulation. The dog possessing intact adrenals, when deprived of food and water and subjected to far more severe stress, readily redistributes his body fluids and maintains normal fluid equilibrium between intra- and extracellular compartments. Although more data than are now available are needed before an adequate explanation can be given to account for this singular behavior of fluid distribution in these stressed dogs, nevertheless certain assumptions are not unwarranted. Presumably there is a rapid increase of osmotically active substances within cells following stress procedures such as metrazol injections, as employed here and electroshock reported by others(5). This seems a reasonable asumption in view of the violent sequela which follow their application; convulsions, muscle rigidity and brief suspension of respiration. Acidosis, due to accumulation of lactic acid, CO_2 and other metabolites undoubtedly occurs, as evidenced in the metrazol experiment by drastic fall in blood pH and plasma bicarbonate (Table III). According to Hamburger and Mathé(6), acidosis contracts the extracellular fluid space. An increase in cellular osmotically active metabolites, hence cell hypertonicity, probably results from intestinal manipulation due to such factors as anesthesia, anoxia, tissue trauma, withdrawal of blood for sampling and severe stimulation of nerves supplying the intestine.

The increased osmolarity of cells with resulting shift of fluid into them could induce severe dehydration of the extracellular space with lowering of plasma volume and arterial pressure to levels incompatible with life. However this may be, the crux of the problem remains, *viz.,* inability of the adx dog not receiving substitution therapy, to redistribute the intracellular fluid, and the ease with which this is accomplished in fasted animals prostrate from either insufficiency or following severe stress, when glucocorticoids are injected. The dramatic correction of the disturbed internal fluid equilibrium in the adx animal by C_{11}-oxy steroids is somewhat reminiscent of the hyperhydration of tissue slices which occurs *in vitro* when these are placed in isosmotic solutions of various kinds and tissue respiration is greatly reduced. A surprising amount of fluid is shifted into the cells markedly increasing their water content(7-11). This fluid and any accompanying ions from the immersion media are held within the cells until the tissue is oxygenated and normal respiration resumed whereupon the extra fluid, ions, etc., are rapidly extruded. Mudge(12), has shown that K is lost by tissues kept in a K free medium and that reaccumulation of this cation from a low external concentration is closely correlated with metabolic activity of the cells as gauged by O_2 consumption. It is possible, although the writers are not aware of substantiating data(13,14), that perhaps the adrenal glucocorticoids may be intimately associated with respiratory or more probably other tissue enzyme systems, which release the energy requisite for reducing the increased cellular osmolarity characteristic of the *adrenalectomized* dog exhibiting insufficiency or in a state of shock after subjection to stress.

Summary. Sensitivity of adrenalectomized dogs to stress induced by intestinal manipulation and metrazol is aparently due to inspissation of the extracellular compartment owing to passage of water into cells. Plasma volume and blood pressure decline, eventuating in circulatory failure. Adrenal glucocorticoids readily release the intracellular fluid and restore the fasted, stressed, dogs to normal. Glucocorticoids may be associated with tissue enzymes which release energy requisite to reduce the cellular hyperhydration characteristic of adrenalectomized dogs exhibiting insufficiency, or shock following stress.

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Digestibility of Corn Germ as Factor in its Apparent Hypocholesterolemic Effects. (24720)

HAROLD A. NASH AND HANS WOLFF (Introduced by Carl A. Bunde)
Pitman-Moore Co., Indianapolis, Ind., and A. E. Staley Manufacturing Co., Decatur, Ill.

Recent reports by Jones(1) and by Stamler(2,3) attribute protective effects against hypercholesterolemia and atherosclerotic lesions to whole corn germ when included in the diet of cockerels. This prompted us to investigate corn germ with the hope of isolating an active principle. Preliminary experiments confirmed apparent activity but raised a question regarding efficiency of digestion of the unground germ. If whole, unground germ is not well digested, comparisons may be made between groups of birds actually absorbing quite different amounts of oil from diets intended to be equivalent in oil content. Since it is standard practice not to include grit in the diet in such experiments, questions of digestibility are particularly pertinent. This report presents investigations of the effectiveness of corn germ and degree to which its oil content is available to the chick.

Methods. Cockerels were raised from birth on chick starter mash fed *ad lib*. They were divided into groups of 10 and placed on experimental diets when about 10 weeks of age and were kept on the diets for 5 weeks. At the end of the test period, blood was drawn for cholesterol determinations. These were run in duplicate on each sample by the method of Pearson(4). The birds were then sacrificed and lesions of the thoracic aorta and brachiocephalic arteries graded according to the scoring system of Tennent(5). Components of

TABLE I. Effects of Experimental Diets on Serum Cholesterol and Atherosclerotic Lesions.

Ration	Source of added lipid	Terminal mean serum cholesterol	Terminal mean atherosclerotic score	% lipid in feces	
Exp. 1					
I	10% refined corn oil	1650 ± 151*	2.08 ± .48*	4.5	
II	20% whole germ #1	834 ± 144	1.71 ± .60	18.5	
III	20% ground germ #1	1420 ± 170	3.21 ± .35	8.3	
IV	21.7% ground germ #2	1700 ± 115	2.17 ± .46	8.2	
Exp. 2					
V	10% refined corn oil	1551 ± 188	1.61 ± .55	3.1	3.3
VI	20% whole germ #1	1173 ± 159	1.94 ± .68	16.3	11.1
VII	20% ground germ #1	1806 ± 215	1.29 ± .45	6.0	6.3
VIII	20% whole germ #1 (1% granite grit in ration)	1306 ± 152	2.0 ± .55	2.4	4.1
IX	21.7% ground germ #2	1963 ± 159	1.95 ± .47	6.3	6.0
X	19.4% ground germ #3	1324 ± 117	1.90 ± .43	6.1	6.2
XI	None	1168 ± 162	1.40 ± .46	3.0	2.9
Exp. 3					
XIIa	10% refined corn oil	2356 ± 257	1.61 ± .39	2.6	
b	*Idem*	2052 ± 178	1.70 ± .39	3.1	
XIIIa	19.4% whole germ #3	962 ± 42	.69 ± .20	17.7	16.6
b	*Idem*	1086 ± 174	.89 ± .39	18.7	17.8
XIVa	19.4% ground germ #3	1975 ± 181	.81 ± .38	5.3	
b	*Idem*	1838 ± 183	1.22 ± .34	5.2	

* ± S.E.

the experimental diets in each case except ration XI were chick starter mash, sucrose, 1% cholesterol and a source of corn lipid. Diets were adjusted to contain 15% protein and 10% added corn lipid. Diet XI differed in that no corn lipid was added. The germ used was prepared by the wet-milling process and was consequently relatively unbroken. Three different lots of germ were compared in the experiments. The sterol content of lot #1 of germ was 0.51% as determined by analysis of petroleum ether extracts made before and after acid hydrolysis. The refined corn oil used in the investigations contained 0.96% sterol. Total lipids in corn germ and feces were determined by exhaustive extraction with 30-60°C petroleum ether in a Soxhlet apparatus of dried and ground samples. There were slight differences in details of first 3 experiments. Cockerels in Exp. 1 were Leghorns of the Hyline Cross and those in Exp. 2 and 3 were of H & N Cross. Ground Wayne Chick Starter* containing 20% protein and 4% fat was used in Exp. 1 and 2. To parallel more closely the rations used by Stamler, chick starter containing 20% protein and 3% fat, manufactured by Kahn Bros. of Chicago, was used in Exp. 3. Exp. 3 differed from the others also in that each ration was fed to 2 different groups of 10 birds in an attempt to assess replicability of results. Exp. 4 was limited to a test of digestibility of whole germ. Fourteen cockerels of 14 weeks of age which had never received grit were placed on a ration similar to that used in the other experiments and containing 20% wet-milled corn germ. After a week on the diet, a record was made of feed consumption for a 48-hour period. Care was taken to recover all uneaten food including that which was dropped on the cage floors by the birds. All fecal droppings were collected during the 48-hour period and weighed after drying. The entire collection was ground and mixed and aliquots were then removed for lipid determination.

Results. Results of feeding experiments may be seen in Table I. In each case feeding of whole germ gave significantly lower serum cholesterol values than were obtained when ground germ was fed. Further, cholesterol values obtained when whole germ was fed are lower in each case than when corn oil was fed although the difference is not statistically sig-

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* Manufactured by Allied Mills, Chicago, Ill.

TABLE II. Recovery of Ingested Fat in Feces (Exp. 4).

Total corn germ consumed in 48 hr (4460 g feed × 20% germ)	892 g
Total corn fat consumed in 48 hr (892 g germ × 43.84% fat)	392
Total fat in 48 hr fecal collection (1920 g feces × 19.75%* fat)	389

* Individual determinations were 20.23, 19.62 and 19.41% fat.

nificant in Exp. 2. The picture is less clear when atherosclerotic lesion scores are considered. Only in Exp. 3 are consistent and significant differences in lesion scores seen. Here the lesions were less severe with a high degree of significance when the whole germ was used as a source of corn lipids (Group XIII) than when corn oil was used (Group XII). The difference between lesion scores for Groups XII and XIV is not significant at the 95% level.

An indication of differences between lots of ground germ is seen in serum cholesterol values for Groups IX and X. Their difference meets tests of significance.

It is important to the interpretation of results that similar blood cholesterol values were seen in group receiving no added oil (Group XI), and the group receiving whole corn germ (Group VI). This is to be expected if little or none of the whole germ is digested. Some measure of the digestion of the germ is to be seen in the fat content of the recovered feces. Little fat was found in the feces when lipid was added to the diet as liquid oil; much when whole germ was used. When ground germ was the source of lipid, fecal fat was low to intermediate in amount. The use of grit in the diet apparently allowed complete digestion of the germ since a low fat content was found in the feces of Group VIII in spite of the fact that the germ was added whole.

Further reinforcing the concept that utilization of the whole germ is very poor in the absence of grit is the observation that large amounts of apparently discrete germ were seen in the fecal matter when such diets were fed. Table II reports a more complete attempt to measure digestion of whole germ added to the diet. The total amount of fat present in the feces was measured over a 48-hour period and was found to nearly equal the fat in the corn germ ingested in the same interval. The nearly exact agreement recorded must certainly have been fortuitous since corrections must be made for unsaponifiables and for incomplete absorption of fat under even the best conditions. Application of a correction based on lipid content of the feces of chicks receiving the same diet but without corn germ (such as diets V, XI) would seem to be the most accurate way of making such allowances. When this is done, the recovery of oil from the ingested corn germ is at least 81%.

Discussion. The results certainly show that a large part of the observed protective effect of whole corn germ is an artifact of incomplete digestion of the germ. At the same time there remain some hints that germ may have a real effect. Among these are: 1) differences between germ lots 2 and 3 as seen in Groups IX and X, 2) failure of germ plus grit (Group VIII) to give as high cholesterol values as ground germ (Group VII), thus suggesting a possible deterioration of an active principle on exposure of the ground germ to air, and 3) lower atherosclerotic lesion scores seen when ground germ was fed rather than refined corn oil in Exp. 3. Of these hints, only the difference between corn germ lots 2 and 3 meets tests of significance.

Cholesterol values in general are higher than those reported by other workers in similar experiments(1,6). Lesion scores are correspondingly high. The reasons for these deviations are not known. Replicability is seen to be good as tested in Exp. 3.

Summary. Digestion of whole germ by chicks not receiving grit is poor. This explains to a large extent the apparent effects of whole corn germ in maintaining low cholesterol levels and preventing atherosclerotic lesions.

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Effect of Spirolactone SC 8109 on Renal Function in Normal Human Subjects.* (24721)

ROY A. WIGGINS, MAXINE E. HUTCHIN, JOHN V. CARBONE[†] AND PAUL D. DOOLAN
Clinical Investigation Center, U. S. Naval Hospital, Oakland, Calif.

A natriuretic effect of synthetic steroids (spirolactones) has recently been shown in the rat, dog(1), and man(2). Kagawa, *et al.* (1) demonstrated that these spirolactones are virtually ineffective in the absence of mineralocorticoid and that effects upon urinary electrolyte excretion are constant so long as a constant ratio of spirolactone/mineralocorticoid is maintained. Similar observations have been reported by Liddle(2) in the human subject. Sodium diuresis has been induced in normal human beings on salt restriction and in patients with aldosterone-secreting adrenal adenoma despite increasing levels of urinary aldosterone(2,3,4). Although Salassa, Mattox, and Power(4) and Liddle(2) have reported that the 24-hour clearance of endogenous creatinine is unchanged in the sodium diuresis induced by spirolactones in man, the literature contains no precise measurements of renal hemodynamics during administration of these substances. The purpose of this study has been to examine more carefully renal function in the normal human subject under the influence of endogenous aldosterone with and without administration of spirolactone.

Materials and methods. The substance used was 3-(3-oxo-17*beta*-hydroxy-19-nor-4-androsten-17*alpha*-yl)propionic acid *gamma*-lactone known by the code name, SC 8109.[‡] Drug administration was at a dosage level of 50 mg each 3 hours beginning at 6:00 to 8:00 a.m. on the day preceding study and continuing until the beginning of the clearance periods. Normal, young, male volunteers were fed a diet containing 20 to 30 meq of sodium, under the supervision of a dietitian. Water and potassium were allowed *ad libitum*. Following 5 to 7 days of sodium restriction, the subjects were studied to determine inulin, para-aminohippurate (PAH) and free water clearances and sodium, potassium and chloride excretion rates. Sodium restriction was continued until the 24-hour urinary excretion rate was in the range observed prior to the first study, then a second set of clearance studies was done. In 3 subjects the control studies were obtained prior to the test, while the remaining 2 were studied in the reverse order. Subjects were studied between 8 a.m. and noon following a 16-hour fast. The men were placed in bed with head elevated to 45° and were not allowed to smoke. A water diuresis was induced by oral administration of 20 ml of water/kilogram of body weight and diuresis was sustained by replacement of urine volume plus estimated unmeasured water loss every 15 minutes. Four control and 4 treatment clearance periods of 15 to 20 min. duration were obtained according to accepted technics of timing and collection of samples(5). Sustaining solutions were administered in a 0.9% solution of sodium chloride at the rate of 3.8 to 4.0 ml/min as controlled by a constant rate infusion pump. Following each experiment, the data were analyzed by Barnard's

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[†] Dept. Med., Univ. Calif. Sch. Med., San Francisco.

[‡] Supplied through courtesy of G. D. Searle & Co., Chicago, Ill.

TABLE I. Renal Function before and during Therapy with Spirolactone (Mean of 4 Observations).

Subjects		ml/min.		Excretion, μeq/min.				μl/min.
		Glomerular filtration rate	Effective renal plasma flow	Na	K	Cl	Urinary Na/K	free water clearance
R.H.	Control	134	642	86	93	30	.64	12.6
	Therapeutic	125	824	209	78	87	2.55	14.1
N.P.	Control	128	706	106	118	29*	.94	11.8
	Therapeutic	117	734	176	64	70	2.67	11.4
S.W.	Control	146	744	69	108	20	.74	10.9
	Therapeutic	143	718	156	60	34	2.75	11.7
C.S.	Control	97	500	120	101	38	1.19	13.4
	Therapeutic	109	511	174	36	52	4.8	14.8
G.S.	Control	149	861	134	154	61	.87	18.6
	Therapeutic	141	831	268	93	112	2.88	19.4

* Mean of 3 observations.

sequential "t" test(6) and experiments discontinued when changes in sodium and potassium excretion were shown to be significant at the 5% level of confidence. Chemical determinations of inulin were performed as described by Roe, Epstein, and Goldstein(7); PAH by Goldring and Chasis(8); chloride by Schales and Schales(9); and sodium and potassium by flame photometry, utilizing a lithium internal standard. Free water clearance was calculated as described by Wesson and Anslow(10). Osmolality determinations were performed cryoscopically on a Fiske osmometer.

Results. Increase in rate of urinary excretion of sodium was observed following administration of the steroid-17-lactone in each of the 5 subjects studied (Table I). Mean control excretion rates were of the order of 69 to 134 μeq/min of sodium. Under the influence of SC 8109 the subjects excreted sodium at a rate of 156 to 268 μeq/min. Experimental values were 1.45 to 2.4 times the control, regardless of the sequence of control and experimental studies. Chloride excretion rates paralleled those of sodium, despite a lower absolute value. Experimental rates were 1.4 to 2.9 times those of the control.

No increase occurred in the glomerular filtration rate or effective renal plasma flow. Indeed, in 4 subjects the clearance of inulin was lower during the drug-induced natriuresis.

Rate of potassium excretion fell significantly during administration of the spirolactone. Following administration of the drug lactone, there was a striking rise in the ratio of urinary Na/K from a mean control value of 0.88 to a mean of 3.1.

An increase in free water clearance was observed in 4 of the 5 subjects during administration of SC 8109. The changes were small (0.8 to 1.5 ml/min) and not statistically significant in this small group.

Discussion. The observation of an increase in urinary sodium and a simultaneous decrease in urinary potassium confirms previous work in the salt-restricted human subject treated with spirolactones. A disparity between the absolute rate of excretion of sodium and chloride is observed; however, the changes in sodium and chloride excretion parallel one another. Landau and Lugibihl(11) have shown a similar disparity between these 2 ions during the presumed mineralocorticoid inhibition by progesterone. The difference has not been explained completely.

The changes in rate of excretion of sodium and potassium reported here are consistent with a withdrawal of aldosterone effect. Several investigators have demonstrated increases in urinary aldosterone in subjects receiving spirolactones. This persistence of urinary aldosterone is not conclusive evidence against a decrease in elaboration of mineralocorticoid but the evidence does suggest that aldosterone production is unchanged by spirolactones. The constant drug/mineralocorticoid ratio necessary to give a constant effect and the lack of effect of spirolactone in absence of mineralocorticoid have suggested that the mechanism of action of the spirolactones might represent competition with mineralocorticoid at a site not yet elucidated. Final acceptance of the thesis of a competitive inhibition must await further study.

This study shows that synthetic steroids do not exert their effect by increasing the filtered load of sodium. The data indicate that sodium diuresis is effected independently of changes in the glomerular filtration rate or effective renal plasma flow.

Summary. Five normal, male, human subjects have been studied by the classical clearance technics during salt deprivation before and after therapy with the steroid-17-spirolactone, SC 8109. No change in the glomerular filtration rate or effective renal plasma flow could be demonstrated but a definite sodium diuresis and potassium retention occurred in response to the spirolactone. A small increase in free water clearance was seen in 4 of 5 subjects studied. These observations localize the effect of spirolactone to the renal tubule.

The assistance of Dr. E. L. Alpen, U. S. Radiological Defense Laboratory, San Francisco, Calif., is gratefully acknowledged in the experimental design of this study.

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Reduction of Post-Occlusive Hyperemia in Cold Subjects.* (24722)

A. C. L. HSIEH

Dept. of Physiology, University of Hong Kong, Hong Kong

Hyperemia that occurs in the forearm following arterial occlusion has been ascribed to anoxia of tissues and accumulation of metabolites(1). This view is supported by experiments of Goldschmidt & McGlone(2) and Jepson(3). However, Folkow(4) found the effect of vasodilator metabolites is slight in cats and suggested that reduction of stretch in the vascular walls during occlusion, resulting in reduction of vascular tone, is the major factor in vasodilation occuring in the first stage of reactive hyperemia. More recently, Patterson(5), in studies of reactive hyperemia in human forearms, has come to similar conclusions. The present experiments were performed to determine the effect of increased vasomotor tone on reactive hyperemia. For this purpose it is assumed that exposure to cold and performance of the cold-pressor test increase peripheral vascular tone.

Methods. Experiments were performed on 2 male subjects in room at about 20°C. The subjects were warm (covered with blanket) or cold (nude). Reactive hyperemia was produced by inflating to 220 mm Hg, a sphygmomanometer cuff wrapped around arm and maintaining pressure for 5 or 10 min. A point on upper forearm, free from veins, was marked and skin temperature recorded every 30 sec. with Stoll-Hardy radiometer accurate to 0.1°C. The results were divided into 4 groups: (W10) subjects warm, occlusion time 10 min.; (W5) subjects warm, occlusion time 5 min.; (C10) subjects cold, occlusion time

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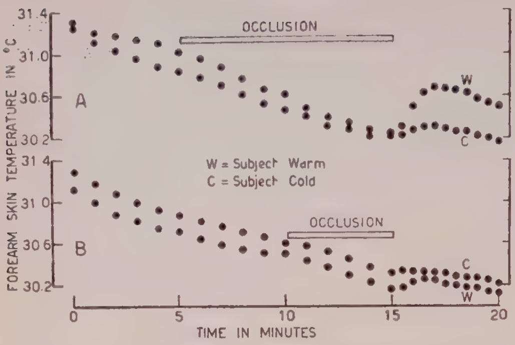

FIG. 1. Forearm skin temperatures before, during and after arterial occlusion. Room temperature, 20°C. Each point is mean of 42 experiments performed on 2 subjects.

10 min.; (C5) subjects cold, occlusion time 5 min. In a few experiments subjects were kept warm and occlusion maintained 10 min. but just before release of cuff pressure, blood flow to contralateral hand was occluded and the hand immersed in water at 4°C.

Results. Temperatures of skin of forearm at time of release of cuff pressure were not significantly different in the 4 groups (Fig. 1). Reactive hyperemia, as measured by change in skin temperature, was greatest in the W10 group, smaller in W5 and C10 groups and insignificant in C5 group (Table I). The difference between temperature responses in W5 and C10 groups was not significant.

TABLE I. Change in Forearm Skin Temperature (°C) 2 Min. after Release of Cuff Pressure.

Occlusion time (min.)	Thermal state of subject	
	Warm	Cold
10	+.48 (p <.001)	+.12 (p <.01)
5	+.08 (p <.01)	+.002 (n.s.)

Each entry is mean of 42 experiments performed on 2 subjects.

When the cold-pressor test was performed on the contralateral hand, temperature response to occlusion was not significant.

Discussion. It is generally known that for a given skin temperature, degree of reactive hyperemia is dependent upon period of occlusion, being greater the longer occlusion is maintained. This has been taken to illustrate the importance of accumulation of metabolites in genesis of reactive hyperemia. Freeman(6) and Freeman and Zeller(7) give evidence suggesting that metabolic needs of tissues determine blood flow through sympathectomized limbs. However, in normal forearms, blood flow is not usually related to need of muscles for oxygen(8). Our observations that the hyperemic response to occlusion can be reduced or even abolished by procedures which increase vasomotor tone strongly suggest that the action of vasodilator metabolites is weak and that centrally controlled vasomotor tone plays an important part in modifying the degree of post-occlusive hyperemia.

Summary. For a given skin temperature and occlusion time, reactive hyperemia in the forearm was reduced or abolished by procedures which increase vasomotor tone.

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Loss of Hemagglutinin on Passage of Vaccinia Virus in L Cells.*† (24723)

WILLIAM A. CASSEL‡ AND BARBARA FATER
Dept. of Microbiology, Hahnemann Medical College, Philadelphia, Pa.

When vaccinia virus was passed serially through Ehrlich ascites tumor, it lost ability to produce hemagglutinin (HA) in embryonated eggs, and capacity to induce formation of HA inhibiting antibodies in rabbits(1,2). Presence of a nonspecific inhibitor of vaccinia HA in cell-free plasma of the Ehrlich tumor (the Ehrlich inhibitor)(3) suggested that this inhibitor might be directly involved in the change of hemagglutinating vaccinia virus to the type of virus which lacks capacity to produce HA. The current experiments concerned with serial passage of vaccinia virus in tissue cultures of L cells strongly indicate the probability of such a situation.

Materials and methods. Virus. Virus designated IHD-E is a neurotropic line of the IHD strain of vaccinia virus, while virus designated IHD-23T is a subline of IHD-E obtained following 23 passages in the Ehrlich tumor. IHD-E produces HA in embryonated eggs, but IHD-23T does not(1). Methods of assaying virus and preparing suspensions for HA and HA-inhibition titrations have been described(1,3,4). *Ehrlich inhibitor.* Cell-free fluids from female NIH mice bearing 13-day Ehrlich ascites tumors (Lettré hyperdiploid line with modal count of 45 chromosomes/cell) served as source of inhibitor. Prior to use, the fluids were subjected to 4 high speed cycles of centrifugation with the fluid decanted from tubes at each cycle. The pooled fluid was then cultured to assure freedom from bacterial contamination, and was stored for a minimum of 6 weeks at –27°C before use(5). When this fluid was thawed for use it received a final centrifugation. Only those fluids showing HA-inhibition titers of 1:80 or greater were employed in tissue culture studies described below. *Tissue culture.* Cultures of the L cell(6,7) were carried in 160 ml pyrex milk-dilution bottles, inoculated at weekly intervals with 1 x 10^6 cells/bottle. The medium consisted of 8.5 ml of medium 199(8) and 1.5 ml of horse serum/bottle. Before use, the horse serum was filtered through Millipore disc, and then heated at 56°C for 30 minutes. Since a nonspecific, heat labile inhibitor was found in certain lots of horse serum, these were carefully avoided.

Virus was passed serially in the medium described above. In addition, virus was passed serially in a medium containing Ehrlich inhibitor. This consisted of 1 ml of a 10X solution of medium 199, 8.5 ml of ascitic fluid containing the Ehrlich inhibitor, and 0.5 ml of horse serum. Five million cells were planted in each bottle, and 4 hours later the virus was inoculated. pH of various preparations was adjusted with phenol red-bicarbonate solution, antibiotics were added to give a final concentration of 200 units of penicillin and 100 μg of streptomycin/ml, and cultures were incubated at 37°C. Control cultures of L cells to which no vaccinia virus was added, but which contained the Ehrlich inhibitor, were employed to eliminate the possibility of contamination with vaccinia virus. Titer of Ehrlich inhibitor in these controls was reduced to one half its original value when the cultures were 6 days old.

Results. When cultures of the L cell in either horse serum medium or Ehrlich inhibitor medium were inoculated with 2 x 10^2 pock-producing particles of virus (ppp), an infectivity peak was reached in 6 days. The titer at this time showed more than a thousand-fold increase in infectious virus. As a matter of convenience, fluids from each virus passage in L cells were harvested at 7 days. Approximately 2 x 10^3 ppp were used to inoculate the L cells in each succeeding passage. From Table I it is apparent that the IHD-E strain of vaccinia virus loses ability to produce HA in embryonated eggs following 17 serial passages in L cells in presence of Ehr-

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‡ Present address: Dept. of Bacteriology, Emory Univ., Atlanta, Ga.

TABLE I. Serial Passage of Vaccinia Virus in Tissue Cultures of L Cells.

	HA titer*			
	Passage in horse serum		Passage in Ehrlich inhibitor	
	Virus		Virus	
Serial TC passage	IHD-E	IHD-23T	IHD-E	IHD-23T
5	40	0†	40	0
10	"	0	80	0
15	"	0	20	0
16			20	
17			0	
18			0	
19			0	
20	80	0	0	0
25	160	0	0	0
30	80	0	0	0

* Hemagglutinin titers of 20% suspensions of chorioallantoic membranes which had been inoculated with 1:10 dilutions of virus-containing tissue culture fluids.

† Designation 0 indicates no hemagglutination at a 1:10 dilution of membrane suspension.

lich inhibitor. In another experiment the hemagglutinating property of the IHD-E virus, passed in L cells in the presence of the inhibitor, was lost after 20 passages.

The results recorded in Table II show that ability to recover the hemagglutinating type of virus is dependent upon number of passages which IHD-E virus has had in L cells in the presence of Ehrlich inhibitor. After 17 tissue culture passages, virus with capacity to produce HA can be recovered after 2 passages in embryonated eggs. On the other hand, after 24 tissue culture passages, the hemagglutinating type of virus could not be recovered after 31 egg passages. The data suggest that the virus with ability to produce HA is gradually replaced by virus which cannot form HA. IHD-E passed in L cells in horse serum medium retains hemagglutinating activity, while IHD-23T retains its inability to produce HA when passed in horse serum or in the Ehrlich inhibitor.

TABLE II. Recovery of Hemagglutinin on Serial Passage of Vaccinia Virus in Embryonated Eggs.

Virus	No. of egg passages required to recover HA
IHD-E 17TC	2
18TC	7
19TC	7
20TC	17
21TC	17
22TC	28
23TC	28
24TC	Not recovered after 31 passages
25TC	*Idem*
26TC	"
27TC	"
28TC	"
29TC	"
30TC	"

Summary. Cell-free ascitic fluids obtained from mice bearing Ehrlich tumor contain an inhibitor which prevents vaccinia virus hemagglutination. Serial passage of the virus in tissue cultures of L cells in presence of the inhibitor favors appearance of virus which has lost ability to produce hemagglutinin. Serial passage of the virus under comparable conditions in the absence of inhibitor did not influence hemagglutinin-producing activity of the virus. These results support the assumption that the loss of hemagglutinin-producing ability by vaccinia virus, on passage through the Ehrlich tumor, results from action of the inhibitor.

We are indebted to Dr. Robert W. Pumper for initial suggestions regarding cultivation of the L cell.

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Serum Lipid Levels in Rats Fed Vegetable Oils with and without Cholesterol.[*] (24724)

COLEMAN R. SESKIND, MARJORIE T. SCHROEDER, RICHARD A. RASMUSSEN
AND ROBERT W. WISSLER
Dept. of Pathology, University of Chicago School of Medicine, Chicago, Ill.

Recent work has pointed to the importance of degree of saturation and fatty acid distribution of dietary fat in affecting serum lipid levels(1-7). The present study was designed to determine the effects of a known atherogenic diet on serum lipid levels in rats with source of fat and presence or absence of cholesterol as the only variables. Two experiments were performed each with a similar plan but each with a different group of dietary lipids to obtain varying levels of saturation and fatty acid content.

Materials and methods. Animals. In 2 experiments 36, 9-month-old albino male Sprague-Dawley rats were marked, weighed, and bled for serum lipid determinations. Animals in first experiment had average weight of 540 g (486-625 g) and had been maintained on Purina laboratory chow. They were divided into 6 groups comparable with respect to distribution of initial weight and serum lipid concentrations. All rats were kept in thermostatically controlled room (76°F), in large wire-bottomed cages with 6 rats/cage. In second experiment rats were 4½ months old and average weight was 424 g (378-456 g).

Diet. A liquid ration, described by Moskowitz(8) of 50% fat, 28% carbohydrate, 5% protein, and 1% choline (on dry weight basis) and containing adequate minerals, vitamins, and roughage, was used. In the first experiment, 6 diets were prepared, each pair contained one of 3 vegetable fats, all presumably free of cholesterol: soybean oil with iodine number (I#) of 129, olive oil (I# 80) and 50% olive oil - 50% palmitic acid mixture (I# 40).[†] Each fat was used to prepare 2 rations one with and one without 1% cholesterol on dry weight basis. Diets were prepared biweekly and fed 3 times daily by stomach tube(9,10) thus eliminating variation in dietary intake. During first week, amount of diet (half of which was water)/feeding was increased gradually from 10 to 15 ml. Each rat then received an estimated 135 calories/day. Animals were allowed tap water *ad lib.* but no additional diet. In second experiment the plan was similar except that a different approach was used to achieve various levels of dietary lipid saturation. Cottonseed oil, commonly used in commercial vegetable shortening, was hydrogenated to levels of saturation similar to those utilized in Exp. 1.[†] Six diets with the same basic composition as Exp. 1 were prepared. The 3 pairs of diets, one with and one without 1% cholesterol, utilized fresh oil (I# 107), hydrogenated oil (I# 78), and laboratory hardened oil (I# 43). *Serum lipids.* Twelve-hour fasting blood samples were obtained from lateral tail vein at biweekly intervals. One ml blood was taken from each rat, pooled with that removed from others of the group, and allowed to clot. The serum was then removed after centrifugation and total cholesterol and lipid phosphorus determined by standard methods(11,12). The baseline, 6 week, and in first experiment, 10 week values were from averaged individual determinations. Animals were all killed at

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[*] This investigation was supported by grant from Nat. Inst. Health, U.S.P.H.S.

[†] Palmitic acid was furnished through courtesy of Dr. Robert J. Vander Wal, Food Research Division, Armour and Co.

These fats were furnished and analyzed by Research Labs. of Swift and Co. *Soybean oil* I# 128.6 Linoleic 41.1, Linolenic 6.4, Arachidonic .00, Oleic 39.8, Saturated Acids 7.4; *Olive oil* I# 81.7 Linoleic 8.6, Linolenic .58, Arachidonic .01, Oleic 71.8, Saturated acids 14.6; *Olive and Palmitic* I# 40 Linoleic 3.39, Linolenic .28, Arachidonic .00, Oleic 38.03, Saturated acids 53.9. *Fresh Cottonseed* I# 109.5, Linoleic 40.1, Linolenic .08, Arachidonic .00, Oleic 41., Saturated acids 18.8, Trans isomers, 1., M.P.–2 to +2°C; *Hydrogenated Cottonseed* I# 77.3 Linoleic 9.27, Linolenic 0.04, Arachidonic .00, Oleic 67.3, Saturated Acids 23.4, Trans isomers 30.9, M.P. 35°C; *Lab Hardened Cottonseed* I# 42.5 Linoleic .0, Linolenic, .0 Arachidonic .0, Oleic 50 (calc.) Saturated Acids 45.6, Trans isomers 28.4, M.P. 50°C.

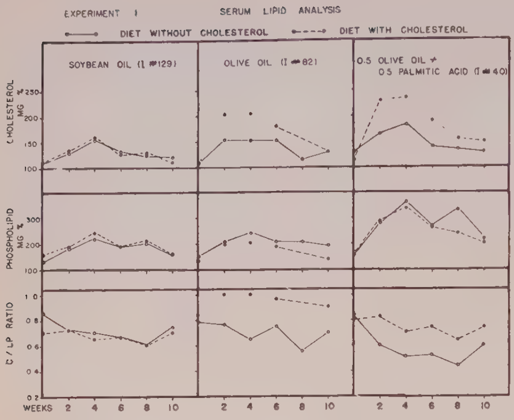

FIG. 1. Serum lipid analysis of Exp. 1.

10 weeks in Exp. 1 and 6 weeks in Exp. 2.

Results of First Experiment. Body Weights. All groups had initial average weight of about 540 g. Both soybean and olive oil groups regardless of whether cholesterol was added, showed similar total weight gains of 200-250 g at end of 10 weeks. On the other hand, rats receiving the most saturated fat consistently showed less weight increase, and at end of experiment this was only one-half that of other groups or about 125 g. Fecal fat excretion study during seventh week with animals in Groups 1 and 5 showed an average 0.3 g more lipid material was excreted/day by rats fed palmitic acid-olive oil ration. Part, if not all, of lesser weight gain may be accounted for by this fact.

Serum Lipid Determinations. Fig. 1 shows results of serum lipid determinations. In every group serum cholesterol rose to a peak at 2-4 weeks, then declined during remainder of experiment even though diets were constant during entire 10 weeks. Groups fed soybean oil (I# 129) showed a slow rise reaching a peak of 160 mg% followed by decline to intial levels whether cholesterol was fed or not. In groups fed olive oil (I# 82), there was a sharper rise to about the same peak level as in preceding groups. When dietary cholesterol was added, there was a definite elevating effect compared to that observed with comparable cholesterol-free ration. Elevations of these serum cholesterol values were maintained longer but at 10 weeks they had declined almost to initial levels. The same phenomena were noted for the 50:50 mixture as for olive oil except that peaks for this ration were the highest attained. Moreover, although a subsequent decrease was noted, serum cholesterols of the group receiving 50:50 fat mixture with added cholesterol did not return to initial levels of blood cholesterol. Serum phospholipid curves resembled cholesterol curves with initial rise and subsequent decline for all diet groups. Soybean and olive oil groups were quite similar. With 50:50 mixture however, phospholipid levels were definitely elevated above levels of other groups. In no instance, however, did addition of dietary cholesterol raise the phospholipid level above its cholesterol-free counterpart as it did with serum cholesterol levels. Phospholipid levels were not elevated until the greatest degree of saturation (or lowest levels of essential fatty acids) had been reached.

With soybean oil diet, serum cholesterol-phospholipid ratio did not change appreciably and addition of cholesterol had no apparent influence. Olive oil alone also had no influence on the ratio, but when cholesterol was added, there was a definite elevating effect. In groups fed the most saturated 50:50 mixture only, the ratio was depressed. Addition of dietary cholesterol again resulted in higher ratio, this time near normal levels. The only apparent correlation between dietary variables and behavior of ratio was observed in relation to oleic acid content of rations.

For cholesterol-free diet groups, increasing saturation of dietary lipid caused increasing liver weights and progressively greater liver percentage of body weights. Moreover, for a given dietary fat, addition of cholesterol resulted in increase of liver weights above those of corresponding cholesterol-free ration.

Results of Second Experiment. Weight Changes. Groups fed the most unsaturated and the moderately saturated cottonseed oil gained weight similarly. Groups fed highly saturated fat declined after first week and showed little net gain at end of experiment. Fecal excretion study for fat at 5½ weeks, on animals in Groups 2 and 6 showed that over 1 g more lipid material was excreted/day by rats receiving laboratory hardened fat. This correlates with their lower body weight.

Serum Lipid Analysis. Fig. 2 shows results

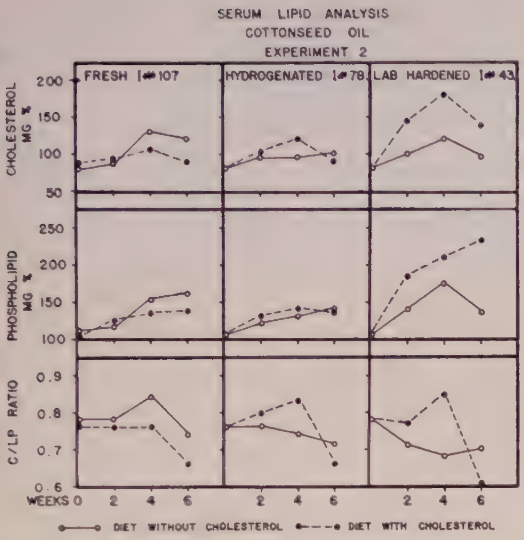

FIG. 2. Mean serum lipid analyses of Exp. 2.

of serum lipid analyses. As in Exp. 1, the general pattern included a peak at 4 weeks and subsequent decline for serum cholesterol values in all groups. In this experiment neither increased lipid saturation nor added dietary cholesterol influenced serum cholesterol level, until dietary fat was very saturated. Then these factors appeared additive. This suggests that a critical degree of saturation (or a threshold level of essential fatty acid deficiency) is necessary to produce elevation of serum cholesterol and to result in further elevation by dietary cholesterol. In almost every case phospholipid values were parallel to serum cholesterol values. The one exception was continued rise with feeding of very saturated fat with added dietary cholesterol. This phenomenon was similar to that observed in the first experiment. Kinsell(7) has reported a similar result in man.

Serum cholesterol-phospholipid ratios paralleled serum cholesterol when either natural or hydrogenated oils were fed. This was also true until 4 weeks, when "lab. hardened" plus cholesterol ration was fed, but at 6 weeks there was a precipitous drop due to continued phospholipid elevation. As in the first experiment the ratio of the group fed the most saturated fat alone was depressed.

Discussion. When one varies saturation of dietary lipids under experimental conditions, other dietary constituents may be changed also. For example such factors as dietary estrogenic substances, sitosterol, and cholesterol content of experimental rations may be inadvertently altered. Furthermore, caloric intake and intestinal absorption may be significant but hidden variables in an otherwise well controlled study. In our experiments care was taken to establish conditions which would allow a valid comparison of effects of saturation of fatty acids with and without added cholesterol on blood lipid levels of the rat. By employing stomach tube feeding technic, and by selected fecal fat analyses, variables of caloric intake and gastrointestinal fat absorption were either well controlled or evaluated. Use of vegetable oils avoided cholesterol contamination of those rations to which it was not added. Employing a biological assay for dietary estrogens(13) ancillary studies in conjunction with the first experiment, indicated that soybean oil had estrogenic activity. Therefore in the second experiment a single fat source, free of estrogenic effect, was employed and degree of saturation was varied by hydrogenation. The sitosterol content of various oils is a variable that was not measured.[‡]

Insofar as our results can be evaluated, it appears that no matter whether saturation of dietary fat is increased by additional palmitic acid or by hydrogenation of cottonseed oil, there is no great tendency for serum cholesterol to rise unless dietary cholesterol is added to the diet. Perhaps of more importance is the observation that dietary cholesterol has little elevating effect on serum cholesterol unless it is combined with highly saturated fat-containing diet. In both experiments the cholesterol-phospholipid ratios were relatively higher when dietary cholesterol was added to the more highly saturated fats.

These results aid in interpretation of several recent reports on relationship between dietary lipids and blood lipids. For example under some dietary conditions there is evidence that dietary cholesterol has little if any effect on blood cholesterol in rats(17). This is said to be the case in man also(4,7,16), but it may be true only when individuals already have an elevated blood cholesterol level or when they receive diets containing considerable unsaturated fat. Although most recent evidence supports the relationship between saturation of dietary fatty acids and elevation of blood cholesterol(3,7), there are also interesting exceptions to this general correlation(14,15). Such variables as amount of fat consumed, initial level of serum cholesterol, length of diet period, and presence or absence of dietary cholate must be evaluated in interpreting these experiments. Furthermore, it is evident from our data that homeostatic factors probably play a major role in influencing ultimate serum cholesterol levels in dietary experiments. In both experiments here summarized, at 2 to 4 weeks, blood lipids of groups receiving the less saturated oils showed differences between groups fed diets with and without dietary cholesterol. These either disappeared or changed at 6 or 10 weeks. It appears, however, that this homeostatic mechanism is least effective when cholesterol is fed with highly saturated lipid.

[‡] Since this manuscript was submitted we have completed sitosterol analyses on the lipids used in this experiment. These are (mg%): Soybean oil .59, Olive oil .38, Cottonseed oil .44. Hydrogenation of cottonseed oil did not alter the sitosterol content. These results indicate that dietary sitosterol was probably not a significant variable in this study.

Although there is evidence suggesting that degree of saturation of dietary fat may influence esterification of serum lipids, especially serum cholesterol(18), further study is necessary to ascertain the relation of these observations to elevation of serum lipids and deposition of lipids in arteries. It appears that among the many factors that influence the level of serum lipids, highly saturated fat and dietary cholesterol combined have a definite additive effect.

Summary. 1) In adult male albino rats tube feeding diets containing large quantities of vegetable fats of differing degrees of saturation and essential fatty acid content, suggests that serum cholesterol is elevated by increasing saturation and that dietary cholesterol augments this effect for the more saturated oils. Elevation appears transient for the less saturated oils but is more sustained for highly saturated fats. 2) Serum phospholipid values in general are parallel to serum cholesterol values. Only with feeding of fats with lowest I# was there an elevation of phospholipid levels to especially high values as compared with serum cholesterol. This effect is particularly striking for "lab. hardened" cottonseed oil fed with cholesterol. 3) Cholesterol/phospholipid ratio shows no difference between groups fed high I# oils with or without dietary cholesterol. The more saturated fats showed elevation of ratios in groups with added cholesterol above groups fed no cholesterol. Highly saturated fat fed alone had a lowering effect on the C/LP ratio.

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Effect of Cyanide on Some Chlorpromazine Responses. (24725)

JACQUES LEBLANC
Dept. of Physiology, Laval University School of Medicine, Quebec City, Canada

We have shown recently that chlorpromazine destroys mast cells *in vivo*(1) and have postulated that some of the hypothermic effects of this substance could be associated with resulting liberation of histamine and serotonine from mast cells(2). After similar studies *in vitro* Hogberg and Uvnas speculated that some of the clinical effects of chlorpromazine may be explained partly by disruption of mast cells(3). On the other hand Junqueira *et al.* have shown that certain substances such as cyanide prevent disruption of mast cells caused by "histamine liberators" (4). In this paper we studied the effect of cyanide on hypothermia and mast cell disruption, normally observed following chlorpromazine injection.

Methods. Male albino rats weighing about 200 g were used. Chlorpromazine* and potassium salts were injected intraperitoneally. In a first experiment, 3 groups of 6 animals were used. After injecting the first group with chlorpromazine (10 mg/kg), the second group with potassium cyanide (0.66 mg/kg), and the third group with chlorpromazine (10 mg/kg) and potassium cyanide (0.66 mg/kg), rectal temperatures were recorded at regular intervals with thermocouple inserted at a depth of 40 mm. In a second experiment, 4 groups of animals received respectively injections of 1) chlorpromazine (10 mg/kg), 2) chlorpromazine (10 mg/kg) and potassium cyanide (0.66 mg/kg), 3) chlorpromazine (10 mg/kg) and potassium chloride (0.75 mg/kg) and 4) saline. Four hours after injection, leucocyte counts were made on blood samples obtained by cutting tip of tail. Finally 24 hours later mast cells of the mesentery were examined as described previously(5).

Results. Fig. 1 shows that although potassium cyanide when used alone has no effect on body temperature, this substance very significantly antagonizes the hypothermic effect normally observed after chlorpromazine administration in rats. Indeed body temperature difference between the group treated with chlorpromazine and the one receiving chlorpromazine and potassium cyanide, is as large as 3°C at third hour after injection.

In addition body temperature returned to normal level in the latter group when it is still almost 2°C lower than normal in the group treated with chlorpromazine alone. Table I also shows the antagonism of potassium cyanide on some of chlorpromazine effect. The destruction of mast cells and leucocytes by chlorpromazine is almost completely prevented by potassium cyanide but not by potassium chloride.

Discussion. Disruption of mast cells by chlorpromazine reported previously(1), is prevented *in vivo* by potassium cyanide. From the work of Motta *et al.*(6), Junqueira *et al.* (4) and the results presented here, it may be said that chlorpromazine and compound 48-80, a powerful histamine liberator, have some common properties. Indeed both substances disrupt mast cells and in both instances this disruption is prevented by cyanide.

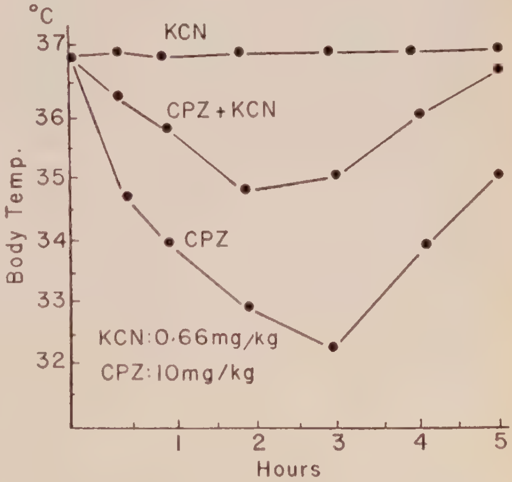

FIG. 1. Effect of chlorpromazine and potassium cyanide on rectal temperatures of rats. (CPZ: Chlorpromazine.)

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* Chlorpromazine was kindly supplied by Poulenc, Ltd., Montreal under trade name Largactil.

TABLE I. Effect of Chlorpromazine and Cyanide on Mast Cells and Leucocytes of Rats.

Treatment*	Mast cells in mesentery/3 mm²	Leucocytes (× 1000)
Saline	310 ± 70†	18.2
CPZ	55 ± 14	11.3
CPZ + KCN	235 ± 80	16.6
CPZ + KCL	50 ± 20	

* CPZ = chlorpromazine (10 mg/kg); KCN = 1.66 mg/kg, and KCL = .75 mg/kg.

† Stand. dev.

In a previous study we postulated that some hypothermic effects of chlorpromazine could be explained by the fact that this substance disrupts mast cells(2). Consequently since cyanide prevents this disruption and also prevents some hypothermia of chlorpromazine, the present study supplies additional evidence to our previous speculation on the possible relationship between mast cell disruption and hypothermia caused by chlorpromazine.

Summary. We have shown that chlorpromazine, like compound 48-80 which is a powerful histamine liberator, destroys mast cells *in vivo* and that cyanide prevents this disruption. Furthermore cyanide also prevents some of the hypothermic effect of chlorpromazine. Consequently, our study may be regarded as further evidence to a possible relationship between mast cell disruption and hypothermia caused by chlorpromazine.

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Histochemical Demonstration of 17-β-Hydroxysteroid Dehydrogenase by Use of Tetrazolium Salt.* (24726)

BJARNE PEARSON AND FRED GROSE
Detroit Inst. of Cancer Research and Dept. of Pathology, Wayne State University College of Medicine, Detroit, Mich.

Talalay has been able to induce specific steroid dehydrogenases in bacteria, and to separate these enzymes into α and β enzymes (1). These include the 3α and 3β and 17β-hydroxysteroid dehydrogenases, which require DPN† as a coenzyme. Farber *et al.*(2) have demonstrated histochemically the distribution of diaphorases within the kidney by means of neotetrazolium chloride. Since hydroxysteroid dehydrogenases require DPN as a coenzyme, the hydrogen transfer goes through a diaphorase intermediate reaction and can therefore be demonstrated histochemically by means of a tetrazolium salt. A fine histochemical localization with permanent slides can be achieved by use of 2,2′-diphenyl-5,5′-di-(*m*-nitrophenyl)-3,3′ - (4, 4′-biphenylene) ditetrazolium chloride introduced by us as substrate for demonstration of dehydrogenases(3). Our object is to demonstrate histochemically 17β-hydroxysteroid dehydrogenase by means of this tetrazole.

Methods. Small blocks of tissue were taken from rats immediately after sacrifice and frozen at –70°C and sectioned at –20°C at 5 μ. The sections were transferred to incubation solution consisting of Tris (hydroxymethyl) aminomethane buffer pH 8.8 60 mM, magnesium chloride 6 mM, DPN 1 mM, 2,2′-diphen-

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† The following abbreviations are used in text. DPN = diphosphopyridine nucleotide; EDTA = Ethylenediaminetetraacetate; DPNH = reduced diphosphopyridine nucleotide; nitro BT = 2,2′-di-p-nitrophenyl-5,5′-diphenyl-3,3′-dimethoxy-4, 4′ - biphenylene)-ditetrazolium chloride.

TABLE I. Formazan Intensities Related to pH in 17β-Hydroxysteroid Dehydrogenase.

pH	Buffer	Estradiol	Testosterone
		(1.25 mM)	
6.57	Phosphate	0	0
7.05		0	1
7.50		1	1
7.60		2	2
7.66	Tris	2	2
8.05		2	2
8.38		3	3
8.80		3	3

yl-5,5′-di-(*m*-nitrophenyl)-3,3′ - (4,4′-biphenylene) ditetrazolium chloride dissolved in N, N-dimethylformamide 0.56%, steroid dissolved in N,N-dimethylformamide 1.25 mM. 17β-hydroxysteroids used were estradiol and testosterone. Sections were incubated at 25°C, 60 minutes, washed in water, rinsed in 80% alcohol for 5 minutes and mounted in glycerogel. The following controls were all negative; estrone plus DPN, DPN without estradiol, estradiol without DPN, and no DPN or estradiol.

Results. Concentration of steroids as substrates which gave maximum formazan intensities was 1.25 mM in final concentration. Lesser concentrations namely, 0.25 and 0.05 mM final concentrations, gave weaker reactions. Concentrations higher than 1.25 mM were not carried out due to insolubility of the steroid. Final concentrations of DPN of 1.9 mM, 1 mM, and 0.5 mM were tried but 1 mM gave best results.

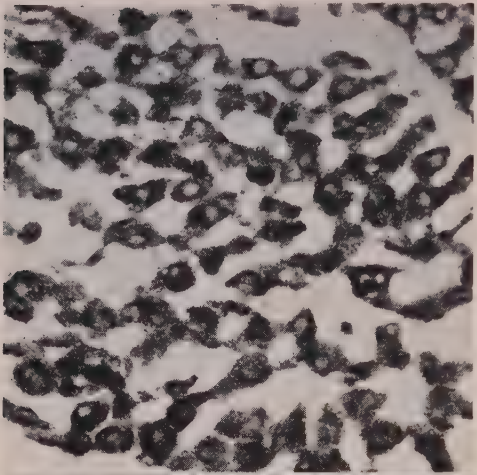
Differences in pH were noted as to intensity of formazan produced. (Table I). Two buffers were used, phosphate and Tris. Histology of the section was better in Tris buffer at higher pH and gave maximum formazan deposition at 8.8.

The effect of several added substances on the 17β enzymes was studied, *viz.;* HgCl$_2$ (5 x 10^{-5}M), KCN (1 x 10^{-3}M), various concentrations of niacinamide from .00 to .03 M final, Zn in the form of sulphate, chloride and acetate (10^{-7}M) and EDTA (3 x 10^{-3}M). Mercuric chloride inhibited completely the reaction of 17β enzyme. Potassium cyanide showed slight inhibition of reaction. Niacinamide gave marked depression of the reaction as did Zn alone and in combination with EDTA and EDTA alone.

Twenty-one different organs and tissues of the rat were tested histochemically for presence of 17β enzyme activity. A large number of male and female rats were used. The following were devoid of demonstrable 17β enzyme activity; kidney, adrenal, spleen, large intestine, lung, skeletal muscle, ovary, testes, uterus, prostate, pancreas, thyroid, thymus, oviduct, cerebrum, cerebellum, lymph node, stomach and heart. The positive organs were liver and small intestine. The entire length of small intestine including duodenum was positive.

To compare activity of 17β-hydroxysteroid dehydrogenase and diaphorase, experiments were carried out using DPNH as substrate for the diaphorase reaction. Using incubation time of 10 minutes and pH 7.6 diaphorase activity was very high in all organs tested with the exception of heart, spleen and brain. The adrenals, small intestine and large intestine gave highest activity as shown by formazan deposition. In contrast to this short incubation time of 10 minutes the 17β-hydroxysteroid dehydrogenase required 60 minutes for maximum reaction and the distribution of formazan was different. Histochemically distribution of 17β-hydroxysteroid dehydrogenase as demonstrated by resultant formazan gave a fine deep blue precipitate within the cytoplasm of the cell. In the liver, only the hepatic cells were involved. Bile ducts, vessels and Kupfer cells were negative (Fig. 1). Enzyme activity was distributed evenly in various portions of the liver lobule, in contrast to succinic dehydrogenase which is absent from areas aroung the central veins(3). In the small intestine 17β-hydroxysteroid dehydrogenase is distributed as fine formazan granules in the cytoplasm of the cell with accentuation of its luminal portion. Nuclei and cells of interstitial tissue were negative (Fig. II).

Discussion. While this work was nearing completion Wattenberg(4) demonstrated 3β-hydroxysteroid dehydrogenase histochemically in tissue sections by use of dehydroepiandrosterone (Δ^5-androstene-3β-ol-17-one) and neotetrazolium and nitro BT. DPN served



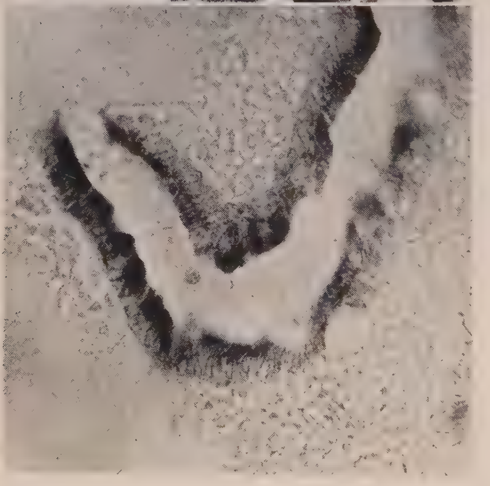


FIG. 1 (top). Liver showing 17 β-hydroxysteroid dehydrogenase activity. Diffuse distribution is present throughout liver cells. 180×.

FIG. 2 (bottom). Small intestine showing 17 β-hydroxysteroid dehydrogenase activity. Enzyme is present in cytoplasm of cells of villi. Stroma is negative. 180×.

as coenzyme and optimum pH was 8.0. All tissues and organs were negative except adrenals, ovary, testes, and liver. This is in contrast to our findings on the 17β-hydroxysteroid dehydrogenase which shows a negative reaction in adrenals, ovaries and testes. Our findings show a positive reaction only in liver and small intestine. Since niacinamide was used in his incubation media we again repeated our results in 17β-hydroxysteroid dehydrogenase with the inclusion of this substance, but found an inhibitory reaction as previously noted.

As estrone is similar to estradiol except that it has a ketone instead of an hydroxyl group in the 17 position, we expected that no dehydrogenation could take place by the action of 17β enzyme in tissue. Estrone was therefore used as control with extensive experiments. No reaction was present in liver and intestine which was positive for the 17β enzyme.

As indicated by Talalay using homogenate material, $HgCl_2$ inhibited almost completely the hydroxysteroid dehydrogenase activity (1). This was also true in our histochemical material. Using homogenates of human placenta, Langer *et al.*(5) demonstrated that Zn ion (10^{-7} to 10^{-3}M) increased the enzymatic activity of estradiol 17β dehydrogenase by as much as 34%. In contrast to this we found a definite inhibition histochemically.

Summary. We demonstrated for the first time the 17β-hydroxysteroid dehydrogenase histochemically by using 2,2′-diphenyl-5,5′-di-(*m*-nitrophenyl)-3,3′-(4,4′-biphenylene) ditetrazolium chloride. Two 17β-hydroxysteroids were used as substrates, *i.e.*, estradiol and testosterone. The optimum pH of the reaction was 8.8 and steroid concentration was determined. There was no apparent difference in activity between the 2 substrates. Controls with estrone in which the 17 group is incapable of dehydrogenation were entirely negative. The liver and small intestine were positive for 17β-hydroxysteroid dehydrogenase. This differs from histochemical results recently reported for steroid 3-β-ol dehydrogenase in that here the organs showing a positive reaction were liver, testes, ovaries and adrenal.

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Mortality of Chick Embryos upon Injection of Homologous Adult Cells.*
(24727)

PAUL I. TERASAKI, JACK A. CANNON AND WILLIAM P. LONGMIRE, JR.
(Introduced by David T. Imagawa)
Dept. of Surgery, University of California Medical Center, Los Angeles

Injection of either adult chicken spleen cells or adult chicken leucocytes into chick embryos caused enlargement of spleens of embryos as well as hemolytic anemia and often death(1,2). Similar results were obtained in mice by Billingham and Brent(3,4) who found that newborn mice injected with spleen cells from adult mice were stunted in growth, possessed atrophied lymph nodes, and often died within 40-60 days after injection. These effects of homologous adult cells in chicks and mice have been interpreted by both groups of workers to be due to adult cells reacting immunologically against the embryonic host. During our studies on induction of tolerance with adult cells to homografts in the chicken, intravenous injection of some types of adult cells caused death. The mortality due to injection of embryonic blood, adult bone marrow, red blood cells, and white blood cells is reported here.

Methods. Various types of cells were obtained from adult chickens or embryos, treated in several ways, and injected intravenously into 14-16 day old chick embryos. Recipient chick embryos and donor chickens or embryos were always of different breeds, New Hampshire or White Leghorn. To obtain *white blood cells,* heparinized blood was centrifuged at 2,500 rpm (International Clinical Centrifuge) for 20 minutes and the buffy coat pipetted off with polyethylene tube (P.E. 260) attached to syringe, bent in half, placed in tube, and re-centrifuged at 4,400 rpm for 20 minutes. This procedure was repeated in a smaller bore polyethylene tubing (P.E. 100) to make final white blood cell suspension. White cells from 6 different donor chickens were tested in varying dosages. *Red blood cells* used for injection were obtained from the lower layer of first centrifugation of blood. *Bone marrow cells* were obtained by anesthetizing donor bird with intravenous injection of sodium pentothal, drilling 2 holes in the femur 3 to 5 cm apart, inserting tight-fitting 18 gauge hypodermic needles attached to two 20 ml syringes. The cells were then centrifuged at 2,500 rpm for 30 minutes, taken out, and recentrifuged in polyethylene tube at 4,500 rpm for 20 minutes. The upper pink colored layer was separated from the lower red layer and the cells suspended in saline for injection. *Embryonic blood* pooled from 26 embryos was taken from embryos incubated 14 or 16 days. An average of 0.7 and 1.0 ml of blood was obtained from 14 day embryos respectively. Upon centrifugation for 20 minutes at 2500 rpm the upper layer of white cells was taken up into a polyethylene tube and recentrifuged for 20 minutes at 4400 rpm. The resulting upper layer of cells and lower layer cells from the first centrifugation were then injected.

Results. White blood cells from adult chickens when injected intravenously into 14-16 day-old chick embryos caused the death of 42 of 52 embryos. Although 10 chicks hatched (19%), only 2 were still alive 2 weeks after hatching. Varying dosages from 600,000 to 106 million white blood cells had roughly similar effects on mortality (Table I). On examination of 36 of the dead embryos, the spleens were enlarged and often had necrotic spots (Fig. 1 and 2). Adult *red blood cells* did not cause as marked an effect on mortality as did adult white blood cells. Twenty-two of the 31 embryos (17%) hatched, and 15 lived at least 2 weeks after hatching. Of the dead embryos examined, 3 of 7 had enlarged spleens. *Bone marrow,* when divided into heavier and lighter centrifugal fractions, showed most of its activity in the lighter fraction. Thus, of chicks injected with cells from the upper layer, 33% hatched as compared to

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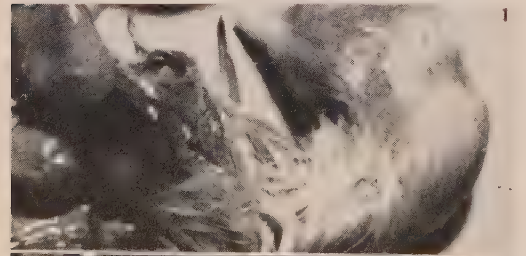
TABLE I. Viability of Chick Embryos following Injection of Various Homologous Cells.

No. of donors	Type of cells & No. of cells $(\times 10^6)$ inj.	No. of eggs inj.	No. chicks hatched	No. chicks hatched & healthy	No. chicks alive at 2 wk	No. of chicks with enlarged spleens/No. of chicks examined
6	Adult white blood cells .6-106 WBC .05-16 RBC	52	10 (19%)	7 (13%)	2 (4%)	36/36
3	Adult red blood cells 32-235 RBC	31	22 (71%)	19 (61%)	15 (49%)	3/ 7
	Adult bone marrow:					
4	Upper centrifugal layer, .3-28 cells	48	16 (33%)	9 (19%)	6 (12%)	17/26
3	Lower centrifugal layer, 2.8-37 cells	33	23 (70%)	20 (60%)	14 (42%)	2/ 8
	Embryonic blood:					
26	Upper centrifugal layer, 3.7-14.3 cells	24	17 (71%)	12 (50%)	8 (33%)	0/ 7
26	Lower centrifugal layer, 6.7-275 cells	31	23 (74%)	20 (64%)	18 (58%)	0/10
3	Plasma—heparinized and citrated	30	30 (100%)	29 (96%)	28 (93%)	0/ 1
1	Serum	10	9 (90%)	8 (80%)	8 (80%)	0/ 1

70% injected with cells from the upper layer, 33% hatched as compared to 70% injected with cells from the lower layer; survival at 2 weeks was 12% as compared to 42%. Embryonic blood, pooled from 26 embryos and centrifuged, did not have a marked effect on rate of hatching. The upper centrifugal layer caused a greater mortality than the lower layer during the period after hatch: 33% lived to 2 weeks of age as compared to 58%. Injection of *plasma* or *serum* had no appreciable effect on mortality.



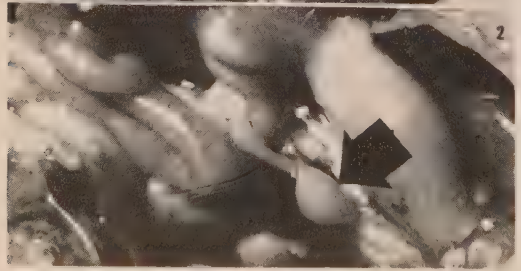


FIG. 1. A representative chick, inj. intrav. at 14 days incubation with white blood cells taken from adult chicken. Death occurred at 20 days incubation with enormous enlargement of spleen, indicated by arrow.

FIG. 2. Arrow indicates spleen of normal untreated chick on 21st day of incubation.

Discussion. Injection of certain types of adult chicken cells into 14-16 day chick embryos of another breed has been found to cause deaths of embryos, usually 5-7 days after injection. This degree of mortality was not obtained by injection of normal contaminants of cell preparations such as serum, heparinized plasma, or citrated plasma. Cells causing from 67-81% of deaths were adult circulating white cells and upper centrifugal layer of bone marrow cells. Invariably embryos injected with these cells die with greatly enlarged spleens. Cells causing 25-30% deaths at time of hatch were the adult red blood cells, lower centrifugal layer of bone marrow cells, and embryonic blood. This percentage of deaths could be considered to be slightly increased over normal experimental mortality since previous studies have shown that a 15-20% mortality could be expected after injection of blood from one embryo(5).

It seems fairly clear then that adult circulating white blood cells can produce death of embryos. Although bone marrow cells produced death, whether marrow cells free of blood white cells can also be effective could not be determined due to the difficulty of obtaining marrow cells free of blood. The small amount of mortality and splenic enlargement due to injection of red blood cells could also be due to contamination with white blood cells.

The types of cells responsible for death seem to agree with the hypothesis of Billingham and Brent(3,4) and Simonsen(1,2) that such deaths are due to "graft *vs* host" reaction in which injected cells consider the embryo to be a homograft and thus react against it. Also consistent with this hypothesis is the fact that embryonic blood cells of the upper centrifugal layer caused delayed mortality when presumably they themselves had matured immunologically in the host.

The possibility that mortality and splenic enlargement is caused by infectious agents has been considered and ruled out by Billingham, Brent, and Simonsen(2,4).

Summary. Circulating white blood cells and the upper centrifugal layer of bone marrow cells taken from normal adult chickens caused a high percentage of deaths when injected into embryonic chicks. Considerably lower percentage of deaths was produced by injection of red blood cells, lower centrifugal layer of bone marrow cells, or embryonic blood.

The authors express appreciation for technical assistance of John McClelland and Mary Ann Coulson.

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Effects of 2-Deoxyglucose on Blood Glucose Levels in the Rat.* (24728)

J. Brown and H. L. Bachrach (Introduced by S. Roberts)
Dept. of Medicine, University of California at Los Angeles School of Medicine

Structural analogues of glucose have proved useful in elucidating some aspects of carbohydrate metabolism. Cramer and Woodward(1) found that 2-deoxyglucose inhibited yeast fermentation and glycolysis of Walker 256 carcinoma, apparently at a site prior to fructose-1, 6-diphosphate cleavage. Sols and Crane(2) observed that brain hexokinase converted 2-deoxyglycose to the 6-phosphate stage but this substance was not a substrate for phosphohexisomerase or glucose-6-phosphate dehydrogenase. Long and Thompson(3) confirmed these findings and suggested that 2-deoxyglucose inhibited hexokinase indirectly by causing accumulation of glucose-6-phosphate. Support for this conclusion was provided by Wick and co-workers(4) who found that 2-deoxyglucose acted as a competitive inhibitor of the phosphoglucoisomerase reaction. Administration of 150 mg/kg of 2-deoxyglucose to eviscerated rabbits reduced intracellular transfer rate of glucose to one-third during 8 hours with maximum insulin stimulation. Insulin accelerated movement of 2-deoxyglucose into rat diaphragms but glucose oxidation was substantially inhibited and fructose oxidation drastically reduced in this system. They proposed accumulation of glucose-6-phosphate with inhibition of hexokinase as the mechanism for inhibition of fructose utilization. The following studies were undertaken to determine the effects of 2-deoxyglucose on some aspects of carbohydrate metabolism in the intact rat.

Methods. Male Sprague-Dawley rats weighing 200-250 g were fasted overnight and anesthetized with sodium pentobarbital. Measure-

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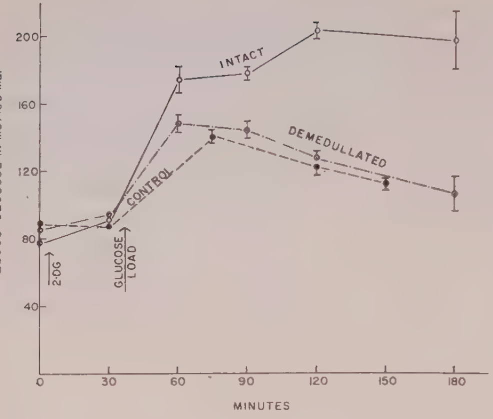

FIG. 1. Blood glucose response to intraper. glucose load (1500 mg/kg) given after 30 min. glucose determination. Effects of 2-deoxyglucose (400 mg/kg) intrav. on intact and demedullated groups compared to saline control.

ment of blood glucose was made in duplicate on 0.2 ml samples of tail blood using the Nelson modification of the Somogyi method(5). 2-deoxy-D-glucose was injected into the exposed saphenous vein in 10% solution 15-30 minutes before the glucose load which was injected intraperitoneally as a 7.5% solution. Injection of deoxyglucose in doses of 400 mg/kg raised blood glucose concentration as determined by this method, less than 10 mg/100 ml at 15-30 minutes after injection and in all cases a second blood glucose determination was made before the glucose load was injected. Adrenal demedullation was performed on 60-80 g male rats at least 4 weeks prior to use to permit cortical regeneration. Adrenals were examined at the end of experiment for completeness of adrenal demedullation. Dihydroergotamine was injected intravenously 15-20 minutes before deoxyglucose at a dose of 1 mg/kg.

Results. Initial experiments were designed to determine effects of injection of varying doses of 2-deoxyglucose on ability of groups of intact anesthetized rats to dispose of a glucose load. Fig. 1 (upper curve) shows results of intravenous injection of 400 mg/kg of deoxyglucose given after initial blood glucose determination, followed by 1500 mg/kg of glucose after second blood glucose determination. These results when compared with those from saline-treated glucose-loaded rats (lower curve) suggest that this dose of deoxyglucose severely inhibited disposition of the glucose load.

Table I reveals blood glucose response of fasted intact rats injected with 400 mg/kg of 2-deoxyglucose after intial glucose determination. A significant rise in glucose concentration compared to controls occurred between 60 and 120 minutes. Adrenal demedullated rats showed no hyperglycemic response to this dose of deoxyglucose, suggesting that hyperglycemia which occurs in intact rats resulted from epinephrine outflow from the adrenal medulla. Intact rats injected intravenously with dihydroergotamine 15 minutes before deoxyglucose also exhibited no response, lending confirmation to the epinephrine hypothesis.

The glucose tolerance curve of adrenal demedullated rats injected first with 400 mg/kg of 2-deoxyglucose followed by a glucose load is shown in Fig. 1. In contrast to the response of intact rats, there was no significant difference between demedullated rats and those given saline followed by a glucose load.

Discussion and Summary. Despite unquestionable evidence from the work of others that 2-deoxyglucose inhibits glucose oxidation in isolated tissues and eviscerated rabbits there was no impairment of disposition of a glucose

TABLE I. Blood Glucose Response in Groups of Fasted Rats to Intravenous 2-deoxyglucose.

		Minutes				
Group	No. rats	0	15	60	120	180
Deoxyglucose, 400 mg/kg	18	90 ± 2*	97 ± 2	99 ± 3	112 ± 5	143 ± 9
Saline controls	13	88 ± 2	79 ± 4	82 ± 2	83 ± 2	82 ± 3
Demedullated rats						
Deoxyglucose, 400 mg/kg	7	84 ± 2		87 ±.2	83 ± 4	82 ± 4
Intact rats						
DHE, 1 mg/kg I.V. after 1st blood	4	74 ± 2	76 ± 2	76 ± 4	76 ± 1	76 ± 3

* Stand. error.

load in adrenal-demedullated anesthetized rats pre-treated with large doses of deoxyglucose. This suggests that other pathways of glucose disposal must be utilized which compensate for inhibition of oxidation. These avenues of glucose disposal will be explored. Inhibition by DHE of the hyperglycemia which results from injection of deoxyglucose into intact fasted rats suggests that this glucose analogue is capable of stimulating release of epinephrine from the adrenal medulla. The lack of hyperglycemia following deoxyglucose injection in adrenal demedullated rats lends support to this hypothesis.

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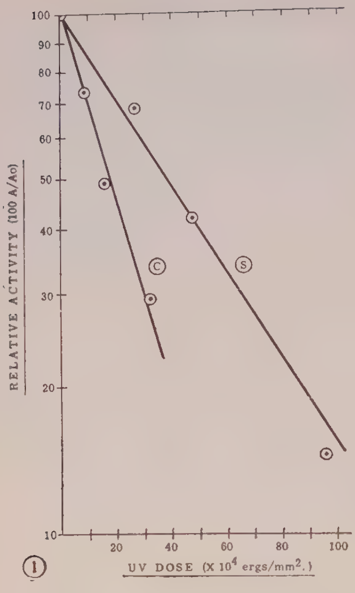
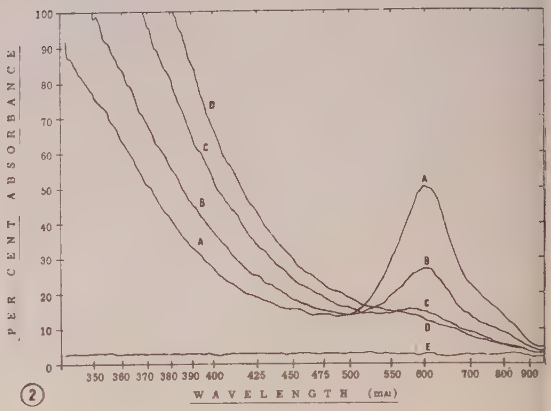
Mechanism of Inactivation of Serum Oxidase (Ceruloplasmin) Activity by Ultraviolet Radiation. (24729)

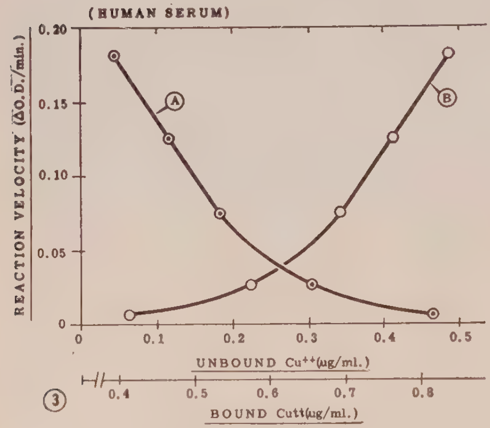
M. H. Aprison and K. M. Hanson (Introduced by D. E. Bowman)
Indiana University Medical Center, Indianapolis

This paper is concerned with the effect of ultraviolet radiation on oxidase activity of ceruloplasmin, the copper containing protein in blood. Ceruloplasmin has a molecular weight of approximately 151,000 and 8 atoms of copper/molecule(2,3). It contains over 90% of copper of blood and is thought to be the copper storage and transport protein. That it also has oxidase activity is interesting. The exact enzymatic role of this copper protein is unknown at present although there have been reports that it can oxidize serotonin(4,5). Data are presented to show that as oxidase activity of serum or ceruloplasmin is destroyed by UV radiation, bound copper in the enzyme is changed resulting in increased amount of "unbound" copper.

Materials and methods. Total copper(6) and direct copper(7) determinations were made by the method of Gubler *et al.* A stainless steel water bath was constructed to hold Petri dish 5 cm in diameter. Ice water at 0°C and not varying more than 0.1° was continuously circulated through water bath. The solution to be irradiated with ultraviolet light was placed in the Petri dish. After temperature equilibration was established, the sample was exposed to UV radiation for definite time interval. A Mineralight model V-41 ultraviolet lamp (Ultraviolet Products, San Gabriel, Calif.) was used as ultraviolet (UV) source. The UV source was mounted above the solution so that it could be moved in vertical or horizontal direction. The lamp was placed 2.5 cm above the solution. At this height the intensity of radiation striking the solution was [1.63 ± 0.09] x 10^4 ergs/minute/mm^2 as determined by uranyloxalate actinometric method(8). Value of quantum yield used in the calculations was 0.60(9). Serum volumes were kept at 6 ml permitting thin uniform layers to be irradiated (3 mm). With purified ceruloplasmin, volumes of 1 ml and 3 ml were used. Evaporation was not noted either with ceruloplasmin or serum. After various samples were irradiated, they were permitted to warm up to 25° C. Aliquots were then analyzed for oxidase activity, total copper, and direct reading copper. Copper free glassware and reagents were used throughout. Serum samples were obtained from staff personnel. Limited amounts of ceruloplasmin were kindly donated by Upjohn Co., Ortho Research Fdn., and Red Cross Blood Program. *Assay.* When N,N-dimethyl-p-phenylenediamine dihydrochloride (DPP) reacts with serum or ceruloplasmin, the substrate is oxidized to a red colored com-







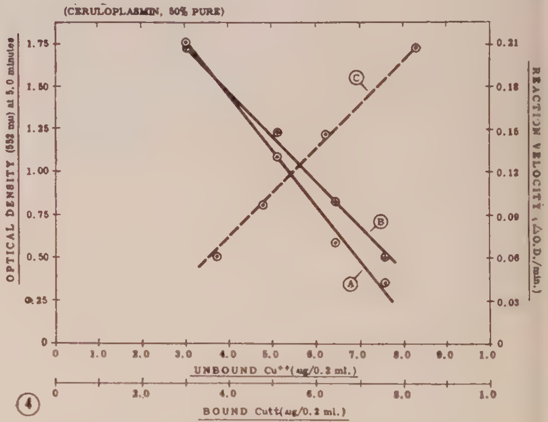


FIG. 1. Effect of UV light (2537 Å) upon ceruloplasmin oxidative activity and human serum oxidase at 0°C. Ordinates: Relative activity ($A\% = 100\ A/A_0$); abscissa: UV dose expressed as ergs/mm². Serum sample (S) contained 87 mg/ml protein and 0.83 μg Cu^{++}/ml in the bound state; the ceruloplasmin sample (C) contained 25.8 mg/ml protein and 41.2 μg Cu^{++}/ml in the bound state.

FIG. 2. Absorbance curves in 340-900 mμ region for 4 ceruloplasmin samples (A-D) exposed to 0, 32.6 × 10^4, 97.6 × 10^4, and 196 × 10^4 ergs/mm² of UV radiation (2537 Å), respectively, and one sample of a $CuSO_4$ solution containing 30 μg Cu^{++}/ml. Before irradiation, ceruloplasmin samples each contained 17.5 μg Cu^{++}/ml in the bound state and 12.1 μg Cu^{++}/ml in the unbound state as well as 10.6 mg/ml protein.

FIG. 3. Relationship between serum oxidase activity and unbound copper (curve A) and bound copper (curve B) in 5 serum samples exposed to different doses of UV radiation.

FIG. 4. Relationship between oxidase activity of human ceruloplasmin (50% pure) and unbound copper (curves A and B) and bound copper (curve C) in 4 samples irradiated with UV light for different lengths of time.

pound(10). Ceruloplasmin and serum oxidase activity was measured by noting change in optical density with time at 552 mμ in a model DU Beckman Spectrophotometer, the cuvette chamber of which was kept at 25 ± 0.1 C by means of thermospacers. Enzymatic activity was expressed either as initial reaction velocity (ΔOD/min or ΔOD/min/μg

Cu^{++}) or as optical density after a given interval of time, such as 5 min. Optical density of serum at 552 mμ was minimal. The blank cuvette contained 1.5 ml distilled water plus 1.5 ml of serum while in the experimental cuvette 1.5 ml of 0.35% DPP solution was added to 1.5 ml serum. The timer was started just as substrate was added. Final pH was 6.2. Under these conditions, the enzyme was assayed at optimum substrate concentration, at optimum pH and at constant temperature (11). Buffer ions which interfere with the reaction(12) were not present. In experiments using purified ceruloplasmin, 1.3 ml of UV irradiated serum (oxidase activity destroyed) plus .2 ml ceruloplasmin were substituted for 1.5 ml serum(13). Absorbance curves of irradiated ceruloplasmin samples were run in model DK-2 Beckman Recording Spectrophotometer.

Results. The effects of UV irradiation on human serum and purified ceruloplasmin kept at 0°C are presented in Fig. 1. The logarithm of relative activity (ordinate) is plotted against corresponding dose of UV irradiation (abscissa). The logarithm of relative activity was proportional to incident energy within experimental error. The results may be represented mathematically by equation 1: $A = A_0 e^{-kD}$(1), where A is oxidase activity after a UV dose D (ergs/mm^2), A_0 is activity of control or nonirradiated sample, and k is a constant. The ratio A/A_0 is the relative activity.

If the irradiation process is continued for sufficiently long period it is possible to inactivate completely the oxidase activity. Of course the thinner the sample layer the shorter the irradiation period necessary to accomplish inactivation.

In experiments in which ceruloplasmin samples were irradiated with UV light, the characteristic blue color changed to lighter shades and the change in intensity of color appeared proportional to amount of radiation absorbed by the solution. The sample receiving enough UV radiation to reduce oxidase activity to less than 5% of normal was light greenish-blue resembling the color of an aqueous solution of cuprous chloride. Absorbance curves in the region 340-900 mμ of 4 ceruloplasmin samples irradiated for 0, 20, 60 and 120 min., respectively, and one sample of cupric sulfate (E) containing 30 μg/ml of Cu^{++} are shown in Fig. 2. Maximum absorbance peak for ceruloplasmin occurred at 600 mμ. Sample A, the non-irradiated control, had the highest absorbance. Sample D, which received a UV dose of 196 x 10^4 ergs/mm^2, showed practically no peak at 600 mμ. Samples B and C fell between A and D in the expected sequence. After passing through their respective maxima, absorbance curves crossed each other in the 500-550 mμ region resulting in complete reversal in the order noted at 600 mμ. This may be due, in part, to greater denaturation of protein material in samples exposed to higher doses of radiation which then could cause a considerable amount of non-specific absorption to occur at the lower wavelengths.

In Fig. 3 data from a typical experiment in which five 6 ml samples of human serum were each exposed to different amounts of UV radiation, are presented to show relationship between resulting fall in enzymatic activity and change in direct reading copper hereafter referred to as "unbound" copper (curve A) and bound copper (curve B) content of samples determined as the difference between total copper and direct reading copper. On curve A, the sample represented by highest enzymatic activity and lowest "unbound" copper is the control which received no irradiation. The other 4 samples, represented by points indicating an increase in "unbound" copper values, received 24.4 x 10^4, 48.9 x 10^4, 97.8 x 10^4, and 196 x 10^4 ergs/mm^2 of UV radiation (2537 Å), respectively. Increase in direct reading copper was inversely proportional to fall in activity in the range in which enzyme activity varied from normal to approximately 30% of normal. Below this point, the change in "unbound" copper was greater than the corresponding fall in oxidase activity. When oxidase activity was plotted against bound copper (curve B), a mirror image of curve A was obtained. The same type of relationship was found when purified human ceruloplasmin was employed in place of serum. The data from such an experiment are shown in Fig. 4. Enzymatic activity was expressed in 2 ways: (1) in curve A, optical density read at 552 mμ 5 min. after the reaction began was plotted against "unbound" copper content, and (2) initial reaction velocity was used as measure of enzyme activity in curve B. The linear relationship between reaction velocity and bound copper is shown by curve C. The difference between the shape of curves presented in Fig. 3 and those in Fig. 4 is that in the case of the experiment with partially purified ceruloplasmin the relationships were linear over the complete range studied.

The effect of —SH inhibitors on serum oxidase was also tested. Mercuric chloride, p-chloromecuribenzoate (sodium salt), o-iodobenzoic acid, and iodoacetic acid (sodium salt), were used in final concentrations of 5 x 10^{-4}M and 5 x 10^{-3}M. Inhibitors were incubated with serum for 30 minutes before the assay began. All showed no significant effect on activity except mercuric chloride. At lower concentrations, an increase in optical density was noted without an increase in rate (O.D. *vs.* time) and this was due to formation of a cloudy reaction upon addition of DPP. At higher concentration, a flocculent precipitate occurred when the substrate was added and the reaction mixture turned a deep red color.

In a limited number of experiments, an attempt was made to reactivate oxidase activity which had been initially destroyed by UV radiation. This was done by incubating the inactivated enzyme with either ascorbic acid plus copper ions or cysteine. All results to date have been negative. In addition, it was not possible to protect adequately the oxidase activity from the effects of UV radiation with cysteine.

Discussion. Apparently the mechanism of inactivation of serum oxidase (ceruloplasmin) is not due to oxidation of –SH groups, but to a change in state of copper in the protein molecule from bound to "unbound." Removal of copper ions by chemical methods also results in loss of enzyme activity(10,14). A similar phenomenon has been shown by Schade and Oyama in the case of siderophilin(15). Its iron binding capacity was inactivated after exposure to UV irradiation.

Recently Heyndrickx(16) has shown that when human serum is exposed to UV irradiation there is an increase in number of –SH groups as measured by the amperometric technic. This as well as the increase in viscosity that is seen when serum is irradiated with UV light, can be taken as indication of unfolding or increase in asymmetry of the protein by splitting of certain bonds or less stable linkages in the molecule. In the case of ceruloplasmin, the decrease in its bound copper content after irradiation could also be explained by assuming rupture of linkages between copper atoms and specific groups of the protein. Apparently ceruloplasmin is a conjugated protein, with copper as an essential part of the molecule, held in a coordination complex or in some similar arrangement. At the present time very little is known in regard to the nature of the chemical bond or linkage between copper and the apoprotein of ceruloplasmin as well as this type of linkage in other copper containing enzymes(17). Although Corwin (18) has given an excellent theoretical review of some of the possible complex configurations in which the copper atom can exist, experimental data are still lacking to show the exact nature of the linkage in copper proteins.

Summary. Oxidase activity of serum and purified ceruloplasmin is inactivated by ultraviolet irradiation at 2537 Å. Loss of enzymatic activity was correlated with a change in state of bound copper of ceruloplasmin to "unbound" copper. When relative enzymatic activity is plotted against radiation dose, the relationship was exponential ($A = A_0e^{-kD}$).

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Use of Pressure Cell to Prepare Cell Walls from Mycobacteria.* (24730)

EDGAR RIBI, THEODORE PERRINE, ROBERT LIST, WILLIAM BROWN,
AND GRANVILLE GOODE (Introduced by Carl L. Larson)
*U. S. Dept. of Health, Nat. Inst. of Allergy and Infectious Diseases,
Rocky Mountain Lab., Hamilton, Mont.*

In a recent report(1) separation of cell wall from internal protoplasm of mycobacteria based on the principle used by Salton and Horne(2) was described. Although yields of cell walls obtained were small, they permitted limited biologic analyses which demonstrated the immunologic significance of this fraction. The demand for larger quantities of cell wall material was met by utilizing a commercial pressure cell in place of the Mickle apparatus (1,2) to achieve selective disruption of organisms. In the resulting cell wall fractions, however, considerable amounts of denatured protoplasmic substances adhered to inner surface of walls and could not be washed off. Further investigation revealed that an increase in temperature at the orifice of the needle valve during bleeding of very highly compressed bacterial suspension apparently caused partial denaturation of protoplasmic components. Since conventional cooling systems were not adequate, an experimental instrument was devised which allowed sufficient cooling of the orifice and needle by exposing them to compressed CO_2 released at a separate needle valve. This paper describes application of such a pressure cell for preparation of cell walls of mycobacteria.

Description and operation of pressure cell. The instrument (Fig. 1) consists essentially of a steel cylinder and piston (A) and 2 needle valve systems (B and C). The outlet at bottom of cylinder is connected by steel tubing to the body of the high pressure needle valve (B) used for bleeding the compressed bacterial suspension. This connection included a high pressure stainless steel nipple, gland nuts, and compression sleeves purchased from American Instrument Co., Silver Spring, Md. The 3-way cross-type stainless steel valve (B) with compression packing was ordered from the same company. Its upper opening (D) is connected by steel tubing to the expansion chamber of the needle valve C (brass body and steel needle) used to bleed compressed CO_2 to cool the needle and orifice of valve B. The lower opening (E) is connected by plastic tubing with suction flask from which glass tubing leads to closed hood equipped with exhaust fan and incinerator. The piston ring, a Parker O Ring No. 2-218, made of compound 49-031, was obtained from Parker Seal Co., Clevland, O. *Operation.* Piston and cylinder were cooled in ice water and the cylinder was then filled with the cold mycobacterial suspension (maximum quantity about 100 ml).

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* We are indebted to Montana Trudeau Society for part of equipment needed.

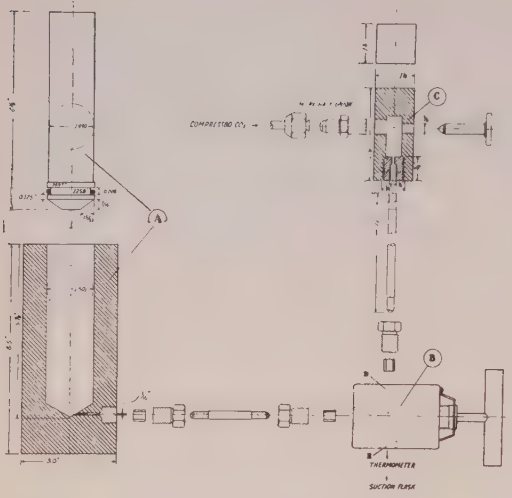

FIG. 1. Diagram of pressure cell used for preparation of mycobacterial cell walls.

Needle valve B was cooled by bleeding CO_2 with valve C until temperature of CO_2 stream, measured near opening E, was maintained at about 2°C. The bacterial suspension was then bled at valve B while a total load of 35,000 to 40,000 lb was maintained on the piston using hand-operated hydraulic press. Valve B was adjusted so that temperature of bacterial products leaving the orifice did not rise above 15°C. Higher temperatures could be avoided either by reducing the flow of the suspension with valve B or by increasing the CO_2 stream with valve C. The suspension was collected in the suction flask which had been placed in ice water bath. The CO_2 vapor, together with aerosol of bacterial material, escaped through the glass tubing to the incinerator.

Preparation of cell walls. Mycobacterium butyricum and 2 strains of *M. tuberculosis*, BCG and H37Ra, were used. The organisms were grown, harvested, and washed as described previously(1). Washed bacteria were dispersed in 0.5% solution of Tween 80 to a concentration of about 3 mg/ml and processed in the pressure cell as described above. All of the following procedures were performed at 4°C the same day the cells were harvested. The mixture resulting from disruption of cells by rapid change of pressure and velocity at orifice was centrifuged at 5000 g for 20 minutes. The supernatant contained dispersed protoplasm, and the sediment consisted of cell walls and unbroken cells. The sediment from each 100-ml portion of starting suspension was resuspended in enough Tween solution to fill three 12-ml conical tubes and centrifuged at 2000 g for one hour. These sediments consisted of a loose, translucent upper layer of cell walls and a hard packed, opaque lower layer of unbroken cells. The cell walls were washed off with Tween solution and further purified by centrifugation and resuspension in distilled water. Electron micrographs showed that the resulting cell walls contained little, if any, protoplasm. Also, whereas cell walls from the Mickle apparatus were usually ruptured at one end of the rod, part of the walls from the pressure apparatus were split perpendicular to the long axes of the cells into a small number of units (Fig. 2). Difficulties encountered during processing disruption products of the Mickle apparatus were not experienced, and nondispersible aggregates were not formed when the pressure cell was used. Therefore, yields of cell walls from a given amount of starting material were much larger. On a dry weight basis, about 60 mg of purified cell walls were obtained per gram of intact cells of the starting material.

Summary. Use of a pressure cell for preparation of purified cell walls of mycobacteria is described. This technic eliminated difficulties encountered earlier with the Mickle pro-

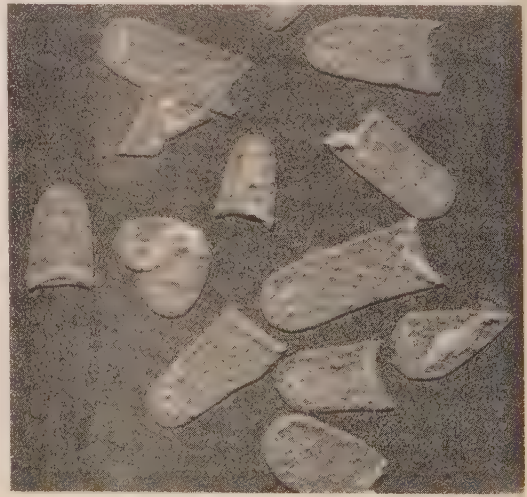

FIG. 2. Cell walls of *Mycobacterium tuberculosis* (BCG). Magnification 64,000 ×.

cedure and the yields were considerably increased.

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Biologic Reactivity of Antigen and Antibody in Specific Precipitate.[*] (24731)

LEON T. ROSENBERG, MARION H. CHANDLER AND EDWARD E. FISCHEL
Dept. of Medicine, Bronx Hospital, N. Y. City

Study of effects of union of antigen with antibody upon specific functional properties of either is of significance in understanding the immune process. Recently, it has been reported that soluble complexes of antigen and antibody can cause immediate reactions which resemble systemic or local anaphylaxis (1,2). Our report(3) indicated that antigen-antibody complexes injected as mixtures in equivalence or in great antigen excess could sensitize local skin sites in guinea pigs so that subsequent challenge by intravenous injection of antigen specifically elicited local anaphylactic reactions. The present study indicates ability of washed specific precipitate, injected intravenously, to sensitize to systemic anaphylaxis when injection of antigen is given subsequently. Furthermore, washed specific precipitate, injected intravenously, acts like antigen to elicit local anaphylactic reactions at skin sites previously sensitized with antibody alone. Thus, the specific biologic reactivity of both antigen and antibody is demonstrated in the complex formed by their union.

Materials. The antigen used was 4 times recrystallized hen egg albumin. Rabbit and guinea pig antisera were analyzed for antibody nitrogen by quantitative precipitin studies(4). Specific precipitate was prepared at equivalence or slight antigen excess as determined by appropriate supernate tests. The precipitate was washed 3 times in cold saline similar to the technic used for preparation of specific precipitate for quantitative estimation of antibody. Washed specific precipitate containing known amounts of antibody and antigen was then suspended with vigorous agitation in chilled saline to make suspensions containing 15 to 140 μg AbN/ml. There was evident variation in particle size of these suspensions. Different preparations of specific precipitate varied somewhat in sensitizing capacity. It has not been determined whether these differences are due to variations between differing antisera or to small differences in ratio of antigen to antibody in the precipitates. The data are presented for ranges of concentration of antibody. The guinea pigs used for systemic or cutaneous anaphylaxis were albino males weighing 250 ± 50 g.

Results. Systemic anaphylaxis. To determine if specific precipitate could sensitize to subsequent challenge injection of antigen, the following experiment was conducted. Guinea pigs were injected intravenously with washed specific precipitate containing from 15 to 140 μg AbN (rabbit). The recipients of these amounts of specific precipitate tolerated the injections well, although with larger amounts of specific precipitate, signs of mild anaphylactic reaction were occasionally observed. At varying intervals thereafter, 1 mg egg albumin was injected intravenously and the animals observed for manifestations of systemic anaphylaxis. Reactions were graded as follows:

0 No reaction; + Chewing, nose scratching, bristling of hair; ++ Coughing, pronouced respiratory embarrassment; +++ Loss of stability, convulsions; ++++ Death. About half of the animals which survived the respiratory component of anaphylaxis showed additional signs of prostration in varying de-

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[*] Aided by grants from N.I.H., P.H.S., Arthritis and Rheumatism Fn. and Helen Hay Whitney Fn.

TABLE I. Systemic Anaphylaxis in Guinea Pigs Sensitized with Washed Specific Precipitate.

μg rabbit AbN inj. i.v. as specific precipitate	Hr between sensitization and challenge 5	24	48–72
15– 30	0, 0, + +, +, ++	0, 0, 0 0, 0, + +, +, +	0, +, + +, ++, ++ ++, ++
70–140	0, +, + +, ++, ++ ++, ++	0, 0, + +, +, + +, +, ++ ++, ++, ++ ++, +++ ++++, ++++ ++++, ++++ ++++	++, +++

Guinea pigs inj. intrav. with suspensions of washed specific precipitate. At intervals indicated, they were challenged by intrav. inj. of 1 mg egg albumin. Degree of typical anaphylactic shock is graded as noted in text.

gree, which differed from those commonly observed in guinea pig anaphylaxis. When severe, these animals were prostrate for as long as one hour. Only severity of the respiratory component, as indicated on the scale above, is presented. Some degree of sensitization was regularly achieved with washed specific precipitate (Table I). Of the 19 guinea pigs injected with 70-140 μg AbN as specific precipitate and challenged with egg albumin 24 hours later, 5 died with signs of typical anaphylaxis. Post mortem findings were also typical of guinea pig anaphylaxis.

The combined antibody was less effective than uncombined antibody in sensitizing to subsequent challenge. Minimum amount of antibody employed, 15 μg AbN, if injected as uncombined antibody, would be expected to sensitize the animals so that those challenged at 24-72 hours would have severe or fatal anaphylaxis(4,6). No animals sensitized with 15 to 30 μg AbN in the specific precipitate showed fatal reactions, although milder manifestations were observed.

Passive cutaneous anaphylaxis (PCA). The following experiment demonstrates that specific precipitate can also act as antigen to elicit PCA. Guinea pigs were sensitized by intracutaneous injection of 0.3 or 0.03 μg AbN of guinea pig anti-ovalbumin. Eighteen hours later 1-1.5 ml of a suspension of thrice washed specific precipitate and 0.5 ml of a 0.5% solution of Evans blue dye (T-1824) were injected intravenously. The reactions of dye localization were graded as follows: 0 No reaction; + 1-5 mm; ++ 6-10 mm; +++ 11-15 mm; ++++ >15 mm dye localization. The results of challenge with specific precipitate are indicated in Table II. Amounts of antigen injected are estimated from analysis of antisera employed to make the precipitate, using an Antibody Nitrogen/Antigen Nitrogen ratio of 10:1. Also indicated in Table II are results obtained using comparable amounts of uncombined antigen. It is evident that small amounts of antigen contained in specific precipitate are able to elicit PCA reactions. Smaller amounts of uncombined antigen sufficed to elicit reactions of

TABLE II. Comparison of PCA Reactions in Sensitized Guinea Pigs Challenged with Washed Specific Precipitate or Small Amounts of Soluble Antigen.

Antigen N contained within specific precipitate (μg)	Reactions
10	++, ++, ++++, ++++
3–5	+++, +++, ++++, ++++, ++++, ++++, ++++
1–1.5	0, +, ++, ++++
.1	0, 0, 0
Soluble antigen N (μg)	
3	++++
1	++++, ++++, ++++
.1	0, +, +, ++, +++, ++++

Guinea pigs were inj. intracut. with 0.3 μg guinea pig AbN, one test site/animal. Eighteen hr later animals were challenged by intrav. inj. of dye and specific precipitate or dye and soluble antigen, and intensity of PCA reactions noted as indicated in text.

equal intensity, and the spot of blue dye appeared somewhat more rapidly in animals challenged with the soluble antigen. When guinea pigs were sensitized with 0.03 μg AbN, positive reactions were not obtained following challenge with specific precipitate.

The supernate recoverable from recentrifuged suspensions of specific precipitate did not contain sufficient antigen to cause reaction at skin sites. Skin sites injected with 1/10 dilutions of normal rabbit serum did not show dye localization when animals were challenged with specific precipitate and dye, nor did injection of dye and uncombined antibody result in dye localization at test or control sites.

Discussion. Our data demonstrate that the constituents of specific precipitate, antigen and antibody, retain their ability to cause specific biologic reactions. Reactivity of antibody in antigen-antibody complexes has recently been demonstrated for passive anaphylaxis locally(3) and is now established for systemic anaphylaxis as well. It is noteworthy that the same specific precipitate, containing minute amounts of antigen, was able to elicit local anaphylaxis in passively sensitized animals.

Ability of constituents of specific precipitate to react like either antigen or antibody alone may be accounted for, in part by studies which indicate that at equivalence the available combining sites on both antigen and antibody are not fully saturated(4,5). In addition, some dissociation of antigen-antibody complex may occur. Two factors may be related to the apparent quantitative differences between previous(3) and present studies. In this report rabbit antibody was used for local sensitization. Sensitization of guinea pig skin with complexes of rabbit antibody prepared in extreme antigen excess had not been demonstrated in contrast to results with guinea pig antibody complexes. Furthermore, the intravenously injected precipitates may fail to reach sites accesible to soluble antibody to elicit specific manifestations of anaphylaxis in the guinea pig.

Summary. The capacity of washed specific precipitate to sensitize guinea pigs so that a subsequent injection of antigen elicits systemic anaphylaxis is demonstrated. Capacity of specific precipitate to act as an antigenic challenge and elicit passive cutaneous anaphylaxis is also shown. Quantitative relationships of the amounts of antigen and antibody involved are noted.

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Influence of Archaic Reproduction Cycle on Sensitivity Response of Rat Dorsolateral Prostate to Sex Hormones.* (24732)

SAMUEL A. GUNN AND THELMA CLARK GOULD (Introduced by W. F. Dunning)
Dept. of Pathology, University of Miami School of Medicine, Coral Gables, Fla.

Previous studies have shown that Zn^{65} uptake by the rat dorsolateral prostate (DLP) reflects high natural zinc content of the gland (1). It was also demonstrated that amount of Zn^{65} taken up by DLP is under hormonal control(2), and represents a functional state of the gland(3). Recent publication conducted throughout the years 1955, '56 and '57 revealed distinct seasonal variations in capacity of DLP to concentrate Zn^{65}. This seasonal pattern was characterized by 2 periods of high Zn^{65} uptake, namely, during February-March

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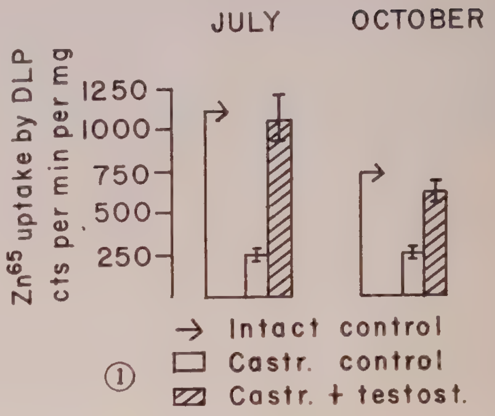
and June-July. Since these periods of peak glandular activity coincided with known breeding seasons of many wild rodents, it was postulated that seasonal pattern of Zn^{65} uptake represented an archaic reproductive cycle inherent in the male laboratory rat(4). The present study was undertaken to ascertain the influence of this archaic breeding cycle on sensitivity response of DLP to administered sex hormones.

Methods. Studies of sensitivity response of DLP to sex hormones were undertaken during 2 periods of the archaic reproductive cycle, namely, during period of high glandular activity (July) and during a phase of low glandular activity (October). Using 16- to 20-week old male Wistar rats sensitivity response was studied in castrates administered 100 μg of testosterone propionate† and in hypophysectomized rats administered 2 units of chorionic gonadotrophin‡ or 200 μg of testosterone propionate. In castrates administration of hormones was begun on day of surgery and continued daily for 6 days. In hypophysectomized rats injection of hormones was begun on 3rd day post-surgery and continued daily for 6 days as outlined previously(3). On 6th day of hormone treatment Zn^{65} was administered by intracardiac injection of 0.04 μc/g. Twenty-four hours later Zn^{65} uptake by DLP was determined by technics described earlier (4). Zn^{65} uptake studies were conducted simultaneously in intact, castrated and hypophysectomized controls. From 6 to 8 animals were used for each experimental group. Identical laboratory conditions were maintained during July and October experiments.

Results. Fig 1 shows that when Zn^{65} uptake by DLP of the intact control rat was high (July), the castrated rat administered testosterone, responded with high Zn^{65} uptake. This is in contrast to low Zn^{65} uptake response in the castrate administered androgen when Zn^{65} uptake of intact control animal was low (October). The difference in response between July and October was statistically significant ($P < 0.01$). Fig. 2 illustrates that the hypophysectomized rat administered chorionic gonadotrophin or testosterone responded with high Zn^{65} uptake in July and low Zn^{65} uptake in October. The difference in response between July and October was statistically significant ($P < 0.01$ for gonadotrophin study and $P < 0.05$ for testosterone study).



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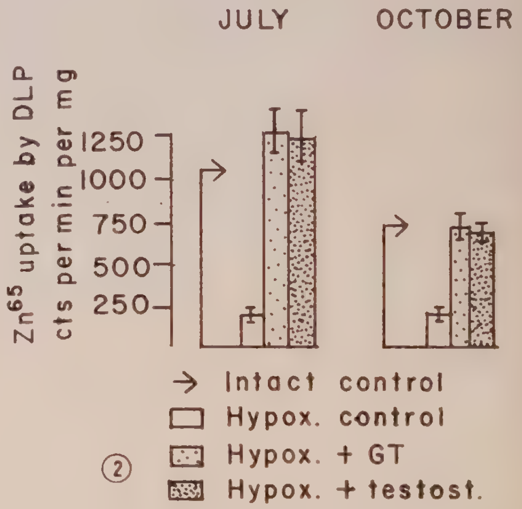

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FIG. 1. Seasonal variations in Zn^{65} uptake by DLP of castrated rats administered 100 μg of testosterone propionate. S.E. of mean are shown.

FIG. 2. Seasonal variations in Zn^{65} uptake by DLP of hypophysectomized rats administered 2 units of chorionic gonadotrophin and 200 μg of testosterone propionate. S.E. of mean are shown.

Discussion. The many variables confronting the wild rodent, such as extremes of climate, availability of food and presence of predatory animals, have been instrumental in producing cyclic habits of reproduction(5). In animals with restricted breeding seasons it is known that there is a seasonal variation in sensitivity response of the reproductive organs to sex hormones(6). In the domesticated laboratory rat, however, which is protected from many variables besetting the wild rodent and is thus able to breed year-round, it would be anticipated that sensitivity response of sex accessory glands to hormones would be relatively uniform throughout the year. On the contrary, our experiments using Zn^{65} uptake of DLP, as indicator of glandular function, have revealed variations in sensitivity response of the gland to sex hormones at different times of year, correlating with the pattern of the archaic reproductive cycle(4). Furthermore, our data demonstrated that these variations in sensitivity response of DLP are inherent in the gland itself, since the same seasonal variations were noted even in the absence of pituitary. In use of hormones experimentally and therapeutically, it appears that cognizance of seasonal variations in sensitivity response would do much to reduce variability of results.

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† Testosterone propionate was generously furnished by Schering Corp. Each daily dose was administered 0.2 ml subcutaneously in sesame oil.

‡ Chorionic gonadotrophin was generously furnished by Parke Davis & Co. Each daily dose was administered subcutaneously 0.2 ml in physiological saline.

Summary. Studies using Zn^{65} uptake by DLP as indicator of glandular function have demonstrated seasonal variations in sensitivity response of castrated and hypophysectomized rats to administered sex hormones. These variations in sensitivity response coincide with the pattern of the archaic reproductive cycle found in intact non-breeding male laboratory rat.

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Role of Vi Antigen in *Salmonella paratyphi* C Infections. (24733)

Adrian D. Mandel, L. S. Baron and Charles E. Buckler
(Introduced by O. Felsenfeld)
Division of Immunology, Walter Reed Army Inst. of Research, Walter Reed Army Medical Center, Washington, D.C.

The dominant role of Vi antigen in determining virulence of *Salmonella typhosa* for mice has been demonstrated by Felix and Pitt(1). This antigen is also highly effective in affording mice active and passive protection against *S. typhosa*(1-3). However, whether Vi antigen of *Salmonella paratyphi* C bears the same relationship to infection by and protection against *S. paratyphi* C in mice has not been fully determined. Kaufmann(4) has shown that an antiserum produced against living Vi-containing *S. paratyphi* C passively protected mice against the mouse-virulent Ty2 strain of *S. typhosa*, thus indicating the close immunological identity of the Vi antigen of *S. typhosa* and *S. paratyphi* C. In addition, Kaufmann(4) assumed that Vi antigen was of no importance in determining virulence of *S. paratyphi C*, since *S. paratyphi* C and *Salmonella hirschfeldi*, which differ antigenically only in absence of the Vi antigen in the latter organism, were of equal virulence. This assumption, nevertheless, did not take into account the fact that *S. hirschfeldi* and *S. paratyphi* C studied by Kaufmann, were obtained from different sources. A more critical evaluation of the role of Vi antigen as virulence factor in *S. paratyphi* C infections of mice is best accomplished by comparison of a Vi negative variant with its Vi containing parent strain. It is our purpose to determine relationship of the Vi antigen of *S. paratyphi* C

TABLE I. Mouse Virulence of Vi and Non-Vi Strains of *S. paratyphi* C.

	Challenge dose					
	5×10^3	5×10^4	5×10^5	5×10^6	5×10^7	5×10^8
Vi form	9/12*	12/20	15/20	16/20	20/20	20/20
Non-Vi form	0/20	2/20	5/20	4/20	17/20	"

* No. of deaths in 21 days/No. of mice inj.

to virulence and immunogenicity in mice, by comparing these 2 activities in a Vi-containing strain of *S. paratyphi* C and in a non-Vi containing variant selected from the Vi culture.

Materials and methods. Cultures. The East Africa strain of *S. paratyphi* C used, was the strain originally studied by Kaufmann. This organism contains the Vi antigen but is O agglutinable. The Vi-negative variant was obtained by selection from a population of Vi forms. *S. typhosa* Ty2, used as a control in certain experiments, is well characterized by its high Vi antigen content. All cultures were maintained in the lyophilized state until ready for use and were checked for their Vi antigen content immediately prior to use. A new ampule was used for each experiment. *Virulence tests.* In all virulence titration, and protection experiments, number of deaths was recorded daily for 3 weeks following challenge. For virulence titrations white female Bagg mice weighing 10 to 12 g were injected intraperitoneally with graded doses of a 6-hour meat extract agar culture suspended in 0.5 ml of saline. Viable counts were performed at time of challenge. Twenty mice were used at each dose level. *Immune sera* were prepared in rabbits by successive daily graded intravenous injections of 18-hour-old suspension of live organisms. Each rabbit received a total of 4 x 10^9 organisms in 5 injections. Ten days following last injection, blood was taken from the heart, the serum removed and stored frozen until used. The antiserum against the Vi form had an O agglutination titer of 1:2560 and a Vi hemagglutination titer of 1:10,240(5). The antiserum against the non-Vi form had an O titer of 1:10,240, and was negative for Vi hemagglutinins. The *S. typhosa* Ty2 antiserum had a Vi hemagglutination titer of 1:2560. *Purified Vi antigen* used was prepared from *Escherichia coli* 5396/38 and was kindly supplied by Doctor M. Webster of Division of Immunology, Walter Reed Army Inst. of Research. Its preparation has been described by Webster, Landy and Freeman(6). *Heat-killed vaccines* were prepared from 18-hour meat extract agar cultures suspended in saline and standardized to contain 1 x 10^9 bacteria/ml. Bacterial suspensions were heated at 56°C for one hour. Acetone-killed and dehydrated vaccine was prepared from *S. paratyphi* C and *S. typhosa* Ty2, according to the method described by Landy(7). The vaccine was rehydrated with distilled water immediately prior to use.

Results. Prior to virulence and protection tests, Vi and non-Vi forms of *S. paratyphi* C were examined in morphological, biochemical and nutritional characteristics. The original Vi form required thymine but mutated to thymine independence at a rate of $1\text{x}10^{-6}$. There was, however, no difference in mouse virulence of the thymine-requiring and thymine-independent Vi forms. The Vi-negative form as isolated was thymine-independent. No other difference could be detected.

Virulence of Vi and non-Vi strains. Virulence titrations were repeated on several occasions with similar results. The results of a typical titration of virulence of Vi and non-Vi forms of *S. paratyphi* C are shown in Table I. At challenge levels below 10^5 organisms, the response of groups of mice was flat and very irregular, as reported by Archer and Whitby (8). Above the 10^5 level there was rather sharp response over a 2-3 log range although even at these levels the response was somewhat irregular. Therefore it was not possible by usual graphic methods to determine the LD_{50} of each strain with any degree of accuracy. However, repeated titrations indicated decreased virulence of the non-Vi forms, roughly estimated about one hundred fold, in those titrations amenable to graphic representation. In all subsequent experiments, the challenge doses were adjusted to anticipate a

TABLE II. Active Immunization of Mice against *S. paratyphi* C.

	Challenge dose		
	S. paratyphi C		*S. typhosa* Ty2
Immunizing agent	Vi form, 3 × 10⁶	Non-Vi form, 4 × 10⁸	1 × 10⁸
S. paratyphi C, Vi form, heat-killed	20/20*	19/20	2/19
S. paratyphi C, non-Vi form, heat-killed	"	"	14/20
Control	16/20	20/20	19/20

* No. of deaths in 21 days/No. of mice inj. Intraper. challenge 10 days following immunization.

mortality of 80-100% of control mice.

Active immunization. The comparative effectiveness of Vi and non-Vi forms of *S. paratyphi* C as active immunizing agents was studied, using heat-killed vaccine of each form. Groups of mice were immunized by intraperitoneal injection of 5 x 10^8 heat-killed cells of either form of *S. paratyphi* C. Ten days later, an equal number of mice from each group was challenged by intraperitoneal injections of Vi or non-Vi forms of *S. paratyphi* C. The data presented in Table II demonstrate lack of protection afforded mice by either heat-killed vaccine.

Passive Immunization. Passive immunity to *S. paratyphi* C infections in mice was studied employing rabbit antisera prepared against the Vi and non-Vi strains of *S. paratyphi* C and *S. typhosa* Ty2 as control. Groups of 60 mice were injected intraperitoneally with 0.1 ml of one of these antisera. One hour later 20 mice from each group were injected intraperitoneally with saline suspension of each strain of *S. paratyphi* C and of *S. typhosa* Ty2. The data shown in Table III demonstrate failure of specific antiserum to protect against either organism. In contrast, when Vi antiserum was present, protection against *S. typhosa* Ty2 was good. This protection of mice against *S. typhosa* Ty2 given by the Vi form *S. paratyphi* C antiserum is in agreement with findings of Kaufmann, and is additional evidence of the presence of Vi antibody in the Vi-form antiserum.

Immunization with acetone-killed vaccine. Since it is known that heat reduces Vi antigen content of bacterial cells, active immunity was studied with acetone-killed and dehydrated vaccine of the Vi form. One half ml amounts of a reconstituted acetone-killed and dehydrated vaccine of the Vi form containing 5 x 10^8 cells were injected intraperitoneally into 20 mice. Ten days later, one half of the mice were injected with the Vi form of *S. partyphi* C and the remainder with *S. typhosa* Ty2 as a control for Vi antigenicity of the *S. paratyphi* C vaccine. The results (Table IV) fail to show better protection of mice by vaccine of maximal Vi antigen content.

Immunization with purified Vi antigen. To provide more definitive data on the role of Vi antigen in active immunity of mice against *S. paratyphi* C, purified Vi antigen was used as immunizing agent. Sixty mice were immunized intraperitoneally with 50 μg of purified Vi antigen derived from *E. coli* 5396/38. Seven days later one group of immunized mice was challenged intraperitoneally with the Vi form of *S. paratyphi* C; one group with non-Vi form, and as a control of immunological activity of the purified Vi antigen, the remaining mice were challenged with *S. typhosa* Ty2,

TABLE III. Passive Immunization of Mice against *S. paratyphi* C.

	Challenge dose		
	S. paratyphi C		*S. typhosa* Ty2
Rabbit antiserum	Vi form, 5 × 10⁵	Non-Vi form, 5 × 10⁸	1 × 10⁸
S. paratyphi C, Vi form	13/20*	19/20	1/20
S. paratyphi C, non-Vi form	15/20	"	12/20
S. typhosa Ty2	"	20/20	1/20
Control	"	19/20	19/20

* No. of deaths in 21 days/No. of mice inj. 0.1 ml antiserum intraper. followed in 1 hr by intraper. challenge.

TABLE IV. Active Immunization with AKD Cells and Purified Vi Antigen.

	Challenge dose				
	S. paratyphi C			*S. typhosa* Ty2	
Immunizing agent	Vi form, 5×10^6		Non-Vi form, 5×10^8	1×10^8	
S. paratyphi C, Vi form, 5×10^8 AKD	7/10*			1/10	
Purified Vi† antigen, 50 μg		8/10	9/10		1/10
Control‡	9/10	"	10/10	9/10	9/10

* No. of deaths in 21 days/No. of mice inj. Intraper. challenge 7 days following immunization.

† From *E. coli* 5396/38.

‡ This table includes 2 separately controlled experiments.

Suitable numbers of non-immunized control mice were challenged at the same time. The results demonstrate that a purified Vi antigen failed to provide active protection of mice against infection with either Vi or non-Vi strain of *S. paratyphi C* (Table IV).

Discussion. Since *S. paratyphi* C (Vi+) and *S. hirschfeldi* (Vi–) have been reported of equal virulence for mice(4), it has been assumed that the Vi antigen plays no part in determining virulence of *S. paratyphi* C. In these experiments, when a Vi-positive and a Vi-negative form of the same strain of *S. paratyphi* C were compared in the mouse, the Vi-negative mutant was of reduced virulence. Unlike *S. typhosa,* loss of Vi antigen did not result in an avirulent form. It must be recognized, nevertheless, that the mutant strain may have suffered a loss of factors other than the Vi antigen, which may also be closely associated with the observed decrease in virulence.

The failure of immunizing agents employed, to protect mice against *S. paratyphi* C is evidence that the Vi antigen was not protective under conditions of these experiments. The protection of mice against *S. typhosa* Ty2, given by the Vi form of *S. paratyphi* C, shows that the Vi antigen of these 2 organisms, qualitatively at least, has the same immunological activity. Passive protection against *S. typhosa* Ty2 by antiserum against the Vi form of *S. paratyphi* C indicates that lack of protection against homologous challenge could not have been due to production of a non-protective or "inactive" Vi antiserum by *S. paratyphi* C. It is evident that the Vi antigen does not play the same role in *S. paratyphi* C infection of mice as in *S. typhosa.*

Archer and Whitby(8) have reported that a Vi vaccine prepared from either living or alcoholized cells afforded mice temporary significant protection against toxic (high) challenge doses of *S. paratyphi* C, and that specific O antigen is similarly effective against a fatal (low) challenge dose. These results, which are at variance with the findings reported here using heat-killed and acetone-killed and dehydrated vaccines or purified Vi antigen, may reflect an effect of the method of vaccine preparation on protection. Experiments designed to furnish more information on this point are presently in progress.

Summary. A comparison was made of virulence and immunogenicity of the Vi form of *S. paratyphi* C and of a non-Vi form selected from the Vi form. Repeated virulence titrations in mice indicate that the Vi-negative mutant is of reduced virulence but does not become avirulent. We have been unable to demonstrate that antibody directed against the Vi antigen plays any role in protecting mice against *S. paratyphi* C infection.

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